PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Ong et al.	Examiner: Stacey MacFarlane
Serial No.	:	10/525,266	
Cnfrm. No.	:	4952	Art Unit: 1649
Filed	:	April 25, 2006	
For	:	GROWTH HORMONE-RELEASING PEPTIDES IN THE TREATMENT OR PREVENTION OF ATHEROSCLEROSIS AND HYPERCHOLESTEROLEMIA	

DECLARATION OF SYLVIE MARLEAU UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Sylvie Marleau, pursuant to 37 C.F.R. 1.132, hereby declare as follows:

1. I am an inventor of the above-identified application.

2. I am currently a Professor of Pharmacy and Pharmaceutical Sciences at the University of Montreal (Montreal, Quebec).

3. I received a Ph.D. in Pharmaceutical Sciences from Université de Montréal in 1990, and B.S. in Pharmacy from Université de Montréal in 1983.

4. The focus of my research activities concern the role of CD36 in regulating the cardiovascular system, and the affects of various mediators of lipids and their role in inflammation. I have published more than 30 articles in these areas.

5. I am presenting this declaration to demonstrate (i) that the prior art recognized a known structure/function relationship among Growth Hormone Related Peptides (GHRPs), both generally and specifically among the subset of GHRPs that lack the ability to induce growth hormone secretion; (ii) that the prior art recognized a known structure/function relationship among CD36 ligands that bind to the hexarelin binding site; and (iii) that the invention can be practiced with other members of this art-recognized subclass of GHRPs that lack the ability to induce growth hormone secretion. These topics are addressed separately below.

Growth Hormone Related Peptides (GHRPs)

6. The known structure-activity relationship of a number of GHRP analogs is discussed in Deghenghi, "Impervious Peptides as GH Secretagogues," *In Growth Hormone Secretagogues*, Ghigo *et al.* (eds.), pp. 19-24 (1999) ("Deghenghi") (copy attached as Exhibit 1). The GHRPs, as a art-recognized family, include a number of small peptides and peptidomimetic compounds that are derived from the prototypical GHRP-6 peptide (*see* Deghenghi at Figure 1). One structural feature shared by preferred members of the class of GHRPs is the replacement of D-Trp at position 2 of GHRP-6 with the more stable D-2-methyl Trp derivative (D-Mrp) or beta-naphthylalanine (D-Nal) (Deghenghi at p. 22). Another structural feature is the prolongation of the chain on the N-terminal side (*id.*). Although not required for activity, many of the GHRPs possess the residues -Phe-Lys or -D-Phe-Lys at the normally C-terminal side, which is amine modified to resist degradation (*see* Deghenghi pp. 20-21).

7. PCT Publ. No. WO 00/29011 to Muccioli *et al.* ("Muccioli") (copy attached as Exhibit 2) expands the known structure/function relationship to include other GHRP analogs. The class of GHRP analogs, as defined in May 2000, was known to include those having the formula:

$$AA^1\text{-}AA^2\text{-}AA^3\text{-}AA^4\text{-Lys-R}$$

where AA^1 is imidazolylacetyl, γ-amino butyryl, isopectinyl, tranexamyl, amino isobutyryl, His-D-Trp, His-D-Mrp, Thr-D-Trp, Thr-D-Mrp, D-Thr-D-Trp, D-Thr-D-Mrp, D-Ala-D-Nal, imidazolylacetyl-D-Trp, imidazolylacetyl-D-Mrp, D-Thr-His-D-Trp, D-Thr-His-D-Mrp, Cys-

Tyr-γ-amino butyryl, Ala-His-Trp, Ala-His-D-Mrp, Tyr-Ala-His-D-Trp, Tyr-Ala-His-D-Mrp, D-Ala-D-Trp, or D-Ala-D-Mrp; AA^2 is Ala, D-Nal, D-Lys, D-Mrp, or D-Trp; AA^3 is D-Nal, D-Trp, Mrp, D-Mrp, Phe, or D-Phe; AA^4 is D-Trp, Mrp, D-Mrp, Phe, or D-Phe; and R is Thr-NH_2, D-Thr-NH_2, or -NH_2. Muccioio indicates at page 5, line 31 that compounds containing D-Mrp are preferred. Muccioio also demonstrates at Figures 1-3 that several of these compounds displace I^{125}-Tyr-Ala-hexarelin.

8. Prior to the priority filing date of the present invention, there was also recognition in the art of a subset of GHRP analogs that lack the ability to induce growth hormone secretion. These GHRP analogs are identified, for example, in U.S. Patent No. 6,025,471 to Deghenghi ("Deghenghi '471") (copy attached as Exhibit 3) and Muccioio (Exhibit 2).

9. One subset of these GHRP analogs that lack the ability to induce growth hormone secretion are characterized by the formula:

A-B-D-Mrp-C-E

where A is H or Tyr; B is a spirolactam, tricyclic or bicyclic structure of the type illustrated at col. 2, lines 7-44 of Deghenghi '471; D-Mrp contains an alkyl group having 1 to 3 carbon atoms, but preferably is methyl; C is Trp-Phe-Lys, D-Trp-Phe-Lys, Mrp-Phe-Lys, D-Mrp-Phe-Lys, Trp-Lys, D-Trp-Lys, Mrp-Lys, D-Mrp-Lys, Ala-Trp-D-Phe-Lys, Ala-Mrp-D-Phe-Lys, Ala-D-Mrp-D-Phe-Lys, D-Lys-Trp-D-Phe-Lys, D-Lys-Mrp-D-Phe-Lys, D-Lys-D-Mrp-D-Phe-Lys, or a tricyclic substituent of the type illustrated at col. 2, lines 53-62 of Deghenghi '471; and E is Lys-NH_2 or -NH_2 (with Lys-NH_2 being preferred when C is the tricyclic structure). As described at col. 1, lines 35-44 of Deghenghi '471, one common feature is the presence of at least one Lys residue and an Mrp residue. That these GHRP analogs lack the ability to induce growth hormone secretion is described in the abstract and at col. 4, line 66 to col. 5, line 2 of Deghenghi '471. As described at col. 5, lines 9-11 of Deghenghi '471, the GH-releasing affect of the peptides was assessed according to known procedures. The binding abilities of several of these compounds is demonstrated in Deghenghi '471 at Figure 1, showing the results of I^{125}-Tyr-Ala-hexarelin displacement study.

10. Mucciolo also identifies at page 9, lines 1-6 (Exhibit 2), six GHRP analogs that are within the scope of the formula listed in paragraph 7 above, but lack the ability to induce growth hormone secretion. As noted in paragraph 9 above, procedures were known in the art for discriminating whether a particular GHRP analog induces GH release.

11. Together, Deghenghi, Mucciolo, and Deghenghi '471 identify dozens of preferred GHRP analogs that induce GH secretion and more than a dozen preferred GHRP analogs lack the ability to induce growth hormone secretion (*see* Deghenghi at Table 1; Mucciolo at page 8, line 4 to page 10, line 12; Deghenghi '471 at col. 3, lines 1-40). Thus, the structural features of these classes of GHRPs and the correlation between their structure and function were known in the art prior to the priority filing date of the present application.

Other CD36 ligands that bind to the hexarelin binding site

12. In addition to the classes of GHRPs noted in paragraphs 6-11 above, other compounds that bind to the hexarelin binding site on CD36 were known prior to the priority filing date of the present application. These include: the polyconal rabbit anti-rat CD36 (A371) antibody generated in our laboratory by using the peptide CD36 (164 to 182) coupled to keyhole limpet hemocyanin as immunogen. The specific anti-CD36 immunoglobulins were purified by affinity on 6% crosslinked agarose coupled to the CD35 (164 to 182) peptide. The CD36/antibody complex was visualized with a peroxidase-linked goat anti-rabbit antibody and chemiluminescent enhancement (*see* Bodart *et al.*, "CD36 Mediates the Cardiovascular Action of Growth Hormone-Releasing Peptides in the Heart" *Circ. Res.* 90:844-849 (2002) (copy attached as Exhibit 4)).

Additional Evidence of Enablement Using EP80318

13. To document whether the anti-atherosclerotic effects of EP80817 could be extended to other structural GHRP analogs that show similar selectivity and binding affinity to CD36, the GHRP analog EP80318 was selected for use. EP80318 has the structure Aib-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2, which is disclosed in Deghenghi '471 at col. 3, line 16. Experiments were performed in male apo$E^{-/-}$ and apo$E^{-/-}$/CD36$^{-/-}$ mice fed an atherogenic diet (D12108, cholate-free AIN-76A semi-purified diet, Research Diets Inc. New Brunswick, NJ).

EP80317 (300 μg/kg), EP80318 (300 μg/kg), or vehicle (0.9% NaCl) were administered by daily subcutaneous injections for 6 (12-18) or 12 (6-18) weeks. As shown in the figures attached hereto in Exhibit 5, chronic treatment with EP 80318 reduced total aortic lesions by 30% ($p < 0.01$) and total plasma cholesterol by 32% ($p < 0.05$) compared to vehicle control, whereas EP 80317 reduced total aortic lesions by 41% and total plasma cholesterol by 27% ($p < 0.05$) compared to vehicle control. In contrast, neither plasma triglycerides (2.1 ± 0.3 mmol/L in EP 80318-treated mice and 2.6 ± 0.2 mmol/L in vehicle-treated mice), nor plasma HDL cholesterol (3.6 ± 0.4 mmol/L in EP 80318-treated mice and 3.8 ± 0.4 mmol/L in EP vehicle-treated mice) were significantly modulated. EP 80318 also reduced aortic lesion areas by 45% ($p < 0.02$) when the treatment was delayed by six weeks. These results confirm that other GHRP analogs can also be used to treat atherosclerosis in patients having multiple risk factors (e.g., poor diet, genetic predisposition).

14. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: September 3, 2009

Sylvie Marleau

Sylvie Marleau

Exhibit 1: **Deghenghi, "Impervious Peptides as GH Secretagogues,"** ***In Growth Hormone Secretagogues*****, Ghigo** ***et al.*** **(eds.), pp. 19-14 (1999)**

Growth Hormone Secretagogues

Basic Findings and Clinical Implications

Edited by

E. Ghigo
M. Boghen
F.F. Casanueva
C. Dieguez

University of Torino, Italy
and
University of Santiago de Compostela, Spain

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ELSEVIER SCIENCE B.V.
Sara Burgerhartstraat 25
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Growth Hormone Secretagogues
Edited by E. Ghigo, M. Boghen, F.F. Casanueva and C. Dieguez

Chapter 3

Impervious Peptides as GH Secretagogues

ROMANO DEGHENGHI

Europeptides, 95108 Argenteuil Cedex, France

Growth hormone is a protein, GHRH and the somatomedins family are peptides and are therapeutically available as such. At the time of this writing, none of the more recent Growth Hormone Releasing Peptides and their non-peptidyl mimetics have been approved for treatment, but it is likely that one or more GH secretagogues will eventually become therapeutic agents. Cyril Y. Bowers, the discoverer of the original GHRP series has reviewed their history (1). Other excellent reviews of this new class of GH Secretagogues have been published (2–4).

PEPTIDES VS NON-PEPTIDE MIMETICS

Following the trailblazer, seminal work of Bowers and Momany, ourselves, and groups from Genentech and Novo Nordisk have developed peptidyl analogues of Bowers' GHRP-6.

In the non-peptidyl series, researchers from Merck Research Laboratories are unquestionably in the lead and their spiropiperidine derivative MK-0677 has been the most studied GHS drug candidate. Other groups from Pfizer and Lilly have disclosed in the patent literature their peptidomimetic GH secretagogues.

Medicinal chemists are therefore divided between those who develop non-peptide ligands for peptide receptors and those who continue to favour peptide analogues as potential drugs. The latter have to face the additional problem of how to conveniently deliver their peptide analogues which are poorly absorbed by the oral route.

One of the reasons why peptides are, with few exceptions, not absorbable orally is because of their vulnerability to proteases and peptidases present in the gastro-intestinal tract. In an attempt to minimize this problem, we developed a series of "impervious peptides", so-called because they are poor substrates to peptidases and proteases. Starting from Hexarelin (5), we have downsized the hexapeptide to obtain (see Table 1) a series of smaller peptides of which the pentapeptide derivative EP 51216 and the tripeptide analogue

TABLE 1

COMPARATIVE ACTIVITY OF POTENTIAL PEPTIDE GH SECRETAGOGUES, 300 µg/kg s.c., IN THE 10 DAY RAT MODEL. GH SECRETION WAS ASSESSED 15 MINUTES AFTER ADMINISTRATION OF THE PEPTIDE

Compound	Structure
Inactive (not different from controls)	
L-164,080	Aib-D-Trp-D-HomoPhe-OEt (3)
EP 251	4-Abz-D-Mrp-AlaTrp-D-Phe-Lys-NH_2
EP 252	2-Abz-D-Mrp-AlaTrp-D-Phe-Lys-NH_2
EP 253	3-Abz-D-Mrp-AlaTrp-D-Phe-Lys-NH_2
EP 254	2-N-Methyl-Abz-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 255	Pip-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 256	D-Pip-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 257	4-Amino-Phe-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 258	D-4-Amino-Phe-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 7459	His-Ala-D-Trp-Ala-Mrp-D-Phe-Lys-NH_2
EP 7460	His-D-Trp-Ala-Mrp-D-Phe-Ala-Lys-NH_2
EP 9399	His-D-Mrp-Ala-Trp-D-Phe-Lys (cyclic)
EP 40894	His-D-Mrp-Ala-Phe-D-Trp-Lys-NH_2
EP 50887	TXM-D-Mrp-D-βNal-Phe-Lys-NH_2
EP 51322	GAB-D-Mrp-D-βNal-NH_2
EP 51343	Aib-D-Ser(Bzl)-D-Mrp-NH_2
EP 60021	D-Mrp-D-Mrp-NH_2
EP 60022	GAB-D-Mrp-D-Mrp-NH_2
EP 60260	D-Mrp-D-Mrp-Phe-NH_2
EP 70683	His-D-Mrp-Ala-Cha-D-Phe-Lys-NH_2
EP 92439	His-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2
EP 92440	His-Ala-D-Trp-D-Lys-Mrp-D-Phe-Lys-NH_2
EP 92441	His-D-Mrp-D-Lys-Mrp-D-Phe-Lys-NH_2
Weakly active (GH range, 30–40 ng/ml)	
EP 51321	GAB-D-Mrp-D-βNal-OEt
EP 60261	D-Mrp-D-Mrp-Mrp-NH_2
EP 60274	GAB-D-Mrp-Mrp-NH_2
EP 60275	D-Mrp-Mrp-NH_2
Active (GH range, 50–90 ng/ml)	
MK-677	Hybrid structure (3)
GHRP-6	His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 (3)
EP 7458	His-D-Trp-Ala-Mrp-D-Phe-Lys-NH_2
EP 40736	D-Thr-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 40931	Thr-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2

(continued)

TABLE 1 (continuation)

Compound	Structure
EP 41616	IMA-D-Mrp-D-Trp-Phe-Lys-NH_2
EP 41617	IMA-D-Mrp-D-βNal-Phe-Lys-NH_2
EP 51390	Aib-D-Mrp-Mrp-NH_2
EP 80161	GAB-D-Mrp-D-Mrp-D-Mrp-Lys-NH_2
Very active (GH range, 100–150 ng/ml)	
Hexarelin	His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
GHRP-2	D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH_2 (1)
G 7039	INIP-D-βNal-D-βNal-Phe-Lys-NH_2 (3)
G 7309	INIP-D-βNal-D-Trp-Phe-Lys-NH_2 (3)
EP 259	IMA-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 41614	INIP-D-Mrp-D-Trp-Phe-Lys-NH_2
EP 41613	INIP-D-Mrp-D-βNal-Phe-Lys-NH_2
EP 50477	GAB-D-Mrp-D-Trp-Phe-Lys-NH_2
EP 50886	TXM-D-Mrp-D-Trp-Phe-Lys-NH_2
EP 51215	GAB-D-Mrp-D-Mrp-Phe-Lys-NH_2
EP 92111	His-D-Mrp-Ala-Trp-D-Phe-Lys-OH
EP 92632	Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
Most active (GH range, 150–250 ng/ml)	
EP 40735	His-D-Mrp-Ala-Trp-D-Phe-Lys-Thr-NH_2
EP 40736	His-D-Mrp-Ala-Trp-D-Phe-Lys-D-Thr-NH_2
EP 40737	D-Thr-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 50885	GAB-D-Mrp-D-βNal-Phe-Lys-NH_2
EP 51216	GAB-D-Mrp-D-Mrp-Mrp-Lys-NH_2
EP 51389	Aib-D-Mrp-D-Mrp-NH_2
EP 71583	Cys-Tyr-GAB-D-Mrp-D-Mrp-Mrp-Lys-NH_2
EP 93183	Tyr-Bpa-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 930497	Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
EP 931829	D-Ala-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2

INIP, isonipecotinyl; IMA, imidazolylacetyl; GAB, γ-amino-butyryl; TXM, tranexamyl = 4-(aminomethyl)-cyclohexanecarbonyl; Mrp, 2-methyl-Trp; Aib, α-aminoisobutyryl; Abz, aminobenzoyl; Pip, pipecolyl; Oic, oxy-indolalanine; Bpa, p-benzoyl-Phe.

EP 51389 have been found to be potent GH secretagogues in the infant rat (6) and in the dog. In the latter species and indeed even in humans, the pentapeptide derivative EP 51216 elicited a GH response when given orally at doses of 0.3 to 0.6 mg/kg.

Oral bioavailability, however, is not only dependent on the "imperviousness" of peptides, or indeed even of non-peptidic molecules. Other important factors are the size of the molecule, its lipid–water partition coefficient and the related propensity of forming hydrogen bonding with the aqueous physiologic environment.

[redacted]

An intriguing possibility is to deliver GH secretagogues by sustained release parenteral devices, such as those successfully employed in the field of LHRH analogues, if the sustained release is compatible with therapeutic efficacy and has an acceptable safety profile.

STRUCTURE–ACTIVITY RELATIONSHIP IN THE HEXARELIN ANALOGUES SERIES

In our 1994 communication (7), we reported our motivation to test, in tryptophan rich peptides, the substitution with the more stable 2-Methyl Trp derivative (Mrp).

Apart from an increased chemical stability, the Mrp substitution was beneficial when a D-Trp was replaced by a D-Mrp, but not when a Trp was substituted with Mrp, at least with the well known GHRP-6 structure (Figure 1):

GHRP-6 : His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 (active)
Hexarelin : His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2 (more active)
EP 7456 : His-D-Trp-Ala-Mrp-D-Phe-Lys-NH_2 (less active)

Figure 1.

This observation seemed to indicate the importance of the unencumbered indole N-H of Trp for receptor binding, confirmed by the inactivity of Oxyindolalanine (Oia) derivative of Hexarelin: His-D-Mrp-Ala-Oia-D-Phe-Lys-NH_2 (EP 70683, mixture of two stereoisomers) compared to Hexarelin in the rat (8), in which the indolic N-H is perturbed by the neighbouring oxygen in position 2 (9).

If we take GHRP-6 as the model prototype Figure 1, our investigations have shown that the D-Trp in position 2 can be advantageously substituted with the more stable, more hydrophobic D-2MeTrp (D-Mrp). Bowers had similarly shown that the DTrp could be substituted with a D-Nal (β-Naphthylalanine) in GHRP-2. Some or total loss of activity, as we have seen, occurs when the Trp in position 4 is replaced with the L-2MeTrp or with Oia, the oxidated form of Trp.

Prolongation of the chain on the N terminal side is compatible with retention and even augmentation of activity (cf EP 930497, EP 93183).

It is unlikely that the same hypothalamic, pituitary or peripheral receptors for which GHRP-6 and similar peptides are ligands, show the same specificity for shorter GHS, such as MK 0677 and EP 51389. There is now evidence (10) that this is indeed the case with some of the shorter GHS being unable to fully displace radioligands such as ^{125}I-Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2.

RESISTANCE TO PROTEASES AND PEPTIDASES

Experimentally the metabolic stability of GHRP-6 (SK&F 110679) or of hexarelin has been confirmed at least in the rat from which more than 50% of these peptides can be recovered unchanged in the bile following their subcutaneous administration. This observation prompted the SK&F group to observe that GHRP-6 "was not designed with metabolic stability in mind [but] it is tempting to speculate that the structural features that are important for receptor binding and pharmacological activity of these peptides may also confer metabolic stability, protecting them from degradation by peptidases" (31). We propose the term *impervious peptides* to describe the metabolic stability characteristic of this series of secretagogues.

The resistance to peptidases and protease of Hexarelin (EP23905), the pentapeptide EP51216 and the tripeptide EP 51389 was measured *in vitro* by incubation at 37°C for one hour in conditions that caused extensive degradation of an LHRH analogue chosen as a reference peptide. The results are summarised in Table 2. This table demonstrates the resistance and high resistance of EP23905 and EP51389 respectively. Not surprisingly, EP51389 is totally resistant because of D amino acids composition. The sensitivity of EP51216 to trypsin and protease is essentially due to the deamidation of the C-terminal amide. Surprisingly, EP 23905 (Hexarelin) is very resistant to these enzymes. Since the primary structure cannot explain this resistance, one can suggest a secondary 'cyclic' structure as having a protective effect.

TABLE 2

	Trypsin	Chymotrypsin	Pepsin	Protease
EP51216	37%	0%	0%	51%
EP51389	0%	0%	0%	0%
EP23905	0%	0%	0%	4.5%

The percentage of degradation is calculated as: 100% of residual peptide.

CONCLUSIONS

The peptide approach to the practical development of GH secretagogues remains a viable one, particularly when such peptides are rendered impervious and are appropriately modified to render them less polar and more absorbable by the oral route. The discovery of peripheral receptors opens new opportunities for medicinal chemists and pharmacologists for the development of organ or tissue specific agents.

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[redacted]

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Exhibit 2: PCT Publ. No. WO 00/29011 to Mucciolo *et al.*

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(71) Applicant: ASTA MEDICA AKTIENGESELLSCHAFT [DE/DE]; An der Pikardie 10, D-01277 Dresden (DE).

(72) Inventors: MUCCIOLI, Giampiero; Piazza Consolata, 10, I-10090 Rivalba (IT). PAPOTTI, Mauro; Str. del Salino, 37, I-10133 Turin (IT). GHIGO, Ezio; Strada Superga, 53, I-10020 Baldissero Torinese (IT). DEGHENGHI, Romano; 106, ch. de Bonde, F-78110 Le Vesinet (FR).

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(54) Title: TREATMENT OF TUMORS BY ADMINISTRATION OF GROWTH HORMONE RELEASING COMPOUNDS AND THEIR ANTAGONISTS

(57) Abstract

A method for treating a tumor in a mammal by administering a growth hormone releasing compound or an antagonist thereof in an amount effective to reduce or inhibit proliferation of tumorigenic cells in the mammal. In particular, the tumors to be treated include lung, mammary, thyroid or pancreas tumors. The preferred compounds are certain peptides that contain methyl tryptophan and lysine units.

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Treatment of tumors by administration of growth hormone releasing compounds and their antagonists

FIELD OF THE INVENTION

The invention relates to a method for reducing the proliferation of carcinoma cells by administration of growth hormone releasing peptides and antagonists thereof.

BACKGROUND OF THE INVENTION

Growth hormone (GH) secretion is regulated by two hypothalamic peptides: GH-releasing hormone (GHRH), which exerts stimulatory effect on GH release and somatostatin which exhibits an inhibitory influence. In the last few years, several investigators have demonstrated that GH secretion can also be stimulated by synthetic oligopeptides termed GH-releasing peptides (GHRP) such as hexarelin and various hexarelin analogs (Ghigo et al., European Journal of Endocrinology, 136, 445-460, 1997). These compounds act through a mechanism which is distinct from that of GHRH (C.Y. Bowers, in "Xenobiotic Growth Hormone Secretagogues", Eds. B.Bercu and R.F. Walker, Pg. 9-28, Springer-Verlag, New York 1996) and by interaction with specific receptors localised in the hypothalamus and pituitary gland ((a) G. Muccioli et al., Journal of Endocrinology, 157, 99-106, 1998; (b) G. Muccioli, "Tissue Distribution of GHRP Receptors in Humans", Abstracts IV European Congress of Endocrinology, Sevilla, Spain, 1998). Recently it was demonstrated that GHRP receptors are present not only in the hypothalamo-pituitary system but even in various human tissues not

generally associated with GH release (G. Muccioli et al., see above (a)).

GHRPs and their antagonists are described, for example, in the following publications: C.Y. Bowers, supra, R. Deghenghi, "Growth Hormone Releasing Peptides", ibidem, 1996, pg. 85-102; R. Deghenghi et al., "Small Peptides as Potent Releasers of Growth Hormone", J. Ped. End. Metab., 8, pg. 311-313, 1996; R. Deghenghi, "The Development of Impervious Peptides as Growth Hormone Secretagogues", Acta Paediatr. Suppl., 423, pg. 85-87, 1997; K. Veeraraganaven et al., "Growth Hormone Releasing Peptides (GHRP) Binding to Porcine Anterior Pituitary and Hypothalamic Membranes", Life Sci., 50, Pg. 1149- 1155, 1992; and T.C. Somers et al., "Low Molecular Weight Peptidomimetic Growth Hormone Secretagogues, WO 96/15148 (May 23, 1996).

SUMMARY OF THE INVENTION

The present invention relates to a method for treating a tumor in a mammal which method comprises administering to a mammal in need of such treatment an effective amount of a growth hormone releasing peptide (GHRP) or an antagonist thereof. Alternatively, the compounds used according to the invention can be defined as growth hormone secretagogues or antagonists thereof. The amounts of these compounds are effective to reduce or inhibit the proliferation of tumorigenic cells in the mammal. In an alternative embodiment, these compounds are specified by the feature that they displace the radioactive marker ^{125}I-Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2 (^{125}I-Tyr-Ala-Hexarelin) from a tumor containing tissue of the mammal.

The compounds disclosed herein exhibit binding to tumorigenic tissue and have been found to act on a specific receptor after administration, thus imparting a decrease in the number of tumorigenic cells. Preferably, treated tumors are lung, mammary, thyroid or pancreas tumors.

The above mentioned compounds include certain known compounds (cf. above), but other compounds useful in the invention are not previously published and include a spirolactam, bicyclic or tricyclic peptidomimetic unit. One common feature for all compounds useful in the invention is that at least one lysine unit is present.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph which illustrates the specific binding of ^{125}I-Tyr-Ala-Hexarelin to membranes from different non-endocrine and endocrine human tumors of various origins.

Figure 2 is a graph which illustrates the ^{125}I-Tyr-Ala-Hexarelin binding to membranes from a non-endocrine lung tumor.

Figure 3 is a graph which illustrates the displacement of ^{125}I-Tyr-Ala-Hexarelin to membranes from non-endocrine lung tumor membranes by various compounds. The ordinate represents binding as a percentage of control (i.e. specific binding in the absence of unlabelled competitor).

Figure 4 is a graph which illustrates the effect of Hexarelin, Ala-Hexarelin, Tyr-Ala-Hexarelin, EP80317 (HAIC-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2), D-(Lys)$_3$-GHRP6 (His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2) and MK0677 (N-[1(R)([1,2-dihydro-1-methanesulfonylspiro-(3H-indole,3,4'-piperidin)-1'yl]-2-(phenylmethoxy)ethyl]-2-amino-methylpropanamide-methanesulfonate) on basal and EGF-stimulated ^3H-thymidine incorporation in human lung carcinoma cells.

Figure 5 is a graph which illustrates the effect of Hexarelin, Ala-Hexarelin, Tyr-Ala-Hexarelin, EP80317, D-(Lys)$_3$-GHRP6 and MK0677 on EGF-stimulated ^3H-thymidine incorporation in human lung carcinoma cells shown as dose responsive curves.

Figure 6 is a graph which illustrates the effect of Hexarelin on human lung carcinoma cell proliferation.

Figure 7 is a graph which illustrates the effect of Hexarelin (a) and Ala-Hexarelin (b) on human breast cancer (T47D) cell proliferation.

Figure 8: Effect of Hexarelin (a) and Ala-Hexarelin (b) on human breast cancer (MDA-MB231) cell proliferation.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In this description, the following abbreviations are used: D is the dextro enantiomer, GH is growth hormone, Mrp is 2-Methyl-Trp, IMA is imidazolylacetyl, GAB is γ-amino butyryl, INIP is isopecotinyl, AIB is amino

isobutyryl, Nal is β-naphthylalanine, TXM is tranexamyl, i.e. 4-(aminomethyl)-cyclohexane carbonyl, D-HNH is D-1,2,3,4,5,6-hexahydro-norharman-3-carboxylate, HAIC is (2S,5S)-5-amino-1,2,4,5,6,7-hexahydro-azepino[3,2,1-hi]indole-4-one-2-carboxylate, ATAB is 2-R-(2β,5β,8β)-8-amino-7-oxo-4-thia-1-aza-bicyclo[3.4.0]nonan-2-carboxylate, and Ala, Lys, Phe, Trp, His, Thr, Cys, Tyr, Leu and Ile are the amino acids alanine, lysine, phenylalanine, tryptophan, histidine, threonine, cysteine, tyrosine, leucine and isoleucine, respectively.

In one embodiment of the invention, useful compounds to be administered are of the general formula I:

$$AA^1\text{-}AA^2\text{-}AA^3\text{-}AA^4\text{-Lys-}R \qquad (I)$$

in which:
AA^1 is IMA, GAB, INIP, TXM, AIB, His-D-Trp-, His-D-Mrp, Thr-D-Trp, Thr-D-Mrp, D-Thr-D-Trp, D-Thr-D-Mrp, D-Ala-D-Nal, IMA-D-Trp, IMA-D-Mrp, D-Thr-His-D-Trp, D-Thr-His-D-Mrp, Cys-Tyr-GAB, Ala-His-Trp, Ala-His-D-Mrp, Tyr-Ala-His-D-Trp, Tyr-Ala-His-D-Mrp, D-Ala-D-Trp, or D-Ala-D-Mrp;
AA^2 is Ala, D-Nal, D-Lys, D-Mrp, or Trp;
AA^3 is D-Trp, D-Nal, D-Trp, Mrp, D-Mrp, Phe, or D-Phe;
AA^4 is D-Trp, Mrp, D-Mrp, Phe, or D-Phe; and
R is $-NH_2$, Thr-NH_2, or D-Thr-NH_2.
The compunds containing a D-Mrp unit are preferred.

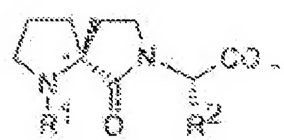
In an another embodiment, the useful compounds include those described in U.S. patent application no. 09/089,954, filed June 3, 1998. These compounds are peptides of the general formula II:

A - B - D-Mrp - C - E (II)

in which:

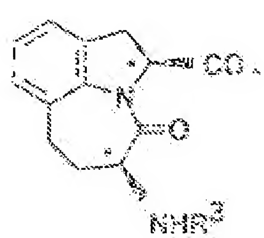
A is H or Tyr;

B is a spirolactam of the general formula III



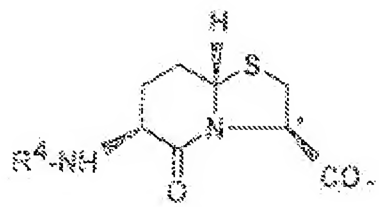
(III)

where R^1 is H or Tyr, R^2 represents the side chain of any one naturally occurring amino acid, and the configuration at * is (R), (S) or a mixture thereof; a tricyclic compound of the formula IV



(IV)

where R^3 is H or Tyr and the configuration at * is (R), (S) or a mixture thereof; a bicyclic compound of the formula V



(V)

where R^4 is H or Tyr and the configuration at * is (R), (S) or a mixture thereof;

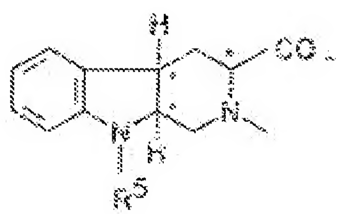
D-Mrp is Dextro-2-Methyl-Trp;

C is Trp-Phe-Lys, D-Trp-Phe-Lys, Mrp-Phe-Lys, D-Mrp-Phe-Lys, Trp-Lys,

D-Trp-Lys, Mrp-Lys, D-Mrp-Lys, Ala-Trp-D-Phe-Lys, Ala-Mrp-D-Phe-Lys,

Ala-D-Mrp-D-Phe-Lys, D-Lys-Trp-D-Phe-Lys, D-Lys-Mrp-D-Phe-Lys,

D-Lys-D-Mrp-D-Phe-Lys, or a tricyclic compound of the formula VI



(VI)

where R^5 is H or SO_2Me and the configurations at * are either (R), (S) or a mixture thereof; and

E is Lys-NH_2 or -NH_2, provided that E is Lys-NH_2, when C is the previously defined tricyclic compound VI.

In accordance with the present invention, it has been found that both GH liberating compounds and compounds that do not liberate GH are useful for the treatment of tumors. Preferably the tumor to be treated according to

the invention is a lung, mammary, thyroid or pancreas tumor.

Specifically preferred GH liberating compounds of the general formula I include the following:

His-D-Trp-Ala-Trp-D-Phe-lys-NH_2,

His-D-Trp-Ala-Mrp-D-Phe-Lys-NH_2,

D-Thr-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

Thr-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

IMA-D-Mrp-D-Trp-Phe-Lys-NH_2,

IMA-D-Mrp-D-Nal-Phe-Lys-NH_2,

GAB-D-Mrp-D-Mrp-D-Mrp-Lys-NH_2,

D-Ala-D-Nal-Ala-Trp-D-Phe-Lys-NH_2,

INIP-D-Nal-D-Nal-Phe-Lys-NH_2,

INIP-D-Nal-D-Trp-Phe-Lys-NH_2,

IMA-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

INIP-D-Mrp-D-Trp-Phe-Lys-NH_2,

INIP-D-Mrp-D-Nal-Phe-Lys-NH_2,

GAB-D-Mrp-D-Trp-Phe-Lys-NH_2,

TXM-D-Mrp-D-Trp-Phe-Lys-NH_2,

GAB-D-Mrp-Mrp-Phe-Lys-NH_2,

Ala -His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

His-D-Mrp-Ala-Trp-D-Phe-Lys-Thr-NH_2,

His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

D-Thr-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

GAB-D-Mrp-D-Nal-Phe-Lys-NH_2,

GAB-D-Mrp-D-Mrp-Mrp-Lys-NH_2,

Cys-Tyr-GAB-D-Mrp-D-Mrp-Mrp-Lys-NH_2,

Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2, and

D-Ala-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

while preferred compounds of the general formula I that do not liberate GH include:

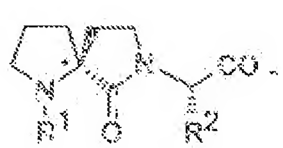
His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2,
His-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,
His-Ala-D-Trp-Lys-Mrp-D-Phe-Lys-NH_2,
His-D-Mrp-D-Lys-Mrp-D-Phe-Lys-NH_2,
His-Ala-D-Trp-Ala-Mrp-D-Phe-Lys-NH_2, and
His-D-Trp-Ala-Mrp-D-Phe-Lys-NH_2.

The preferred compounds of the general formula II include the following:

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Trp-Phe-Lys-NH_2,
[S,S-Spiro(Pro-Leu)]-D-Mrp-Mrp-Lys-NH_2,
[S,S-Spiro(Pro-Leu)]-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,
[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,
Tyr-[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,
[S,S-Spiro(Pro-Ile)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,
[S,S-Spiro(Pro-Leu)]-D-Mrp-D-HNH-(SO_2CH_3)-Phe-Lys-NH_2,
HAIC-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2, and
ATAB-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

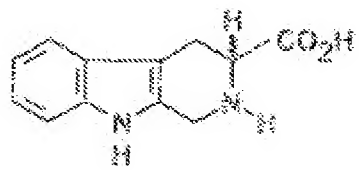
where S,S-Spiro(Pro-Leu) and S,S-Spiro(Pro-Ile) is 4-Methyl-2S[6'-oxo-(5'-S)1',7'-diazaspiro[4,4]nonan-7'-yl-]pentanoate and 3-Methyl-2S[6'-oxo-(5'-S)1',7'-diazaspiro[4,4]nonan-7'-yl-]pentanoate, respectively.

These units have the formula



(III)

where R^1 is H and R^2 is the side chain of Leu or Ile (see P. Ward et al., J. Med. Chem., 33, 1848 (1990)). Also, the tricyclic compound HNH is obtained by conventional hydrogenation of the corresponding tetrahydro-norharman-3-carboxylic acids of the formula



(VII)

The units according to the formulas III, IV, V and VI constitute peptidomimetic units which are advantageous in that they lock in a β-turn configuration thus mimicking natural amino acids.

Pharmaceutically acceptable salts of these compounds can be also used, if desired. Such salts include organic or inorganic addition salts, such as hydrochloride, hydrobromide, phosphate, sulfate, acetate, succinate, ascorbate, tartrate, gluconate, benzoate, malate, fumarate, stearate or pamoate salts.

All compounds can be conveniently synthesized according to the usual methods of peptide chemistry, such as by solid-phase peptide synthesis, as described by E. Atherton and R.C. Sheppard in "Solid Phase Peptide Synthesis", IRL Press at Oxford University Press, 1989, by solution-phase synthesis as described by J. Jones in "The Chemical Synthesis of Peptides", Clarendon Press, Oxford 1994, or by a combination of both solid- and solution-phase methods, as known in the art.

The solid-phase synthesis starts from the C-terminal end of the compounds. A suitable starting material can be prepared, for example, by attaching the required protected α-amino acid to a chloromethylated resin, a hydroxymethylated resin, a benzhydrylamine resin (BHA), or to a para-methyl-benzhydrylamine resin (p-Me-BHA). As an example, an available chloromethylated resin is BIOBEADS SX1 by BioRad Laboratories, Richmond, California. The preparation of the hydroxymethylated resin is described by Bodansky et al., Chem. Ind. (London), 38, 15997 (1966). The BHA resin is described by Pietta and Marshall, Chem. Comm., 650 (1970), and is commercially available by Peninsula Laboratories Inc., Belmont, California.

After the starting attachment, the protecting group of the α-amino acid can be removed by means of different acid reagents, such as trifluoroacetic acid (TFA) or hydrochloric acid (HCl) dissolved in organic solvents at room temperature. After the removal of the protecting group of the α-amino acid, the remaining protected natural amino acids or carboxylic acids corresponding to the units according to the general formulas III, IV, V and VI, which also constitute amino acids, can be coupled step by step in the desired order. Each protected amino acid can generally be reacted in excess of about three times using a suitable carboxyl activating group, such as dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC) dissolved, for example, in methylene chloride (CH_2Cl_2), dimethylformamide (DMF) or their mixtures. After the desired aminoacidic sequence has been completed, the

desired compound can be cleaved from the supporting resin by treatment with a reagent such as hydrogen fluoride (HF) which cleaves not only the compound from the resin, but also the protecting groups of lateral chains. When a chloromethylated resin is used, treatment with HF leads to the formation of a compound which has a terminal acid group and is present in free form. When a BHA or p-Me-BHA resin is used, the treatment with HF directly leads to the formation of a compound which has a terminal amide group and is present in free form.

Medicaments useful for treating tumors in a mammal, including a human, can comprise a compound according to the present invention or a pharmaceutically acceptable salt thereof, or combinations of compounds according to the present invention or pharmaceutically acceptable salts thereof, optionally in admixture with a carrier, excipient, vehicle, diluent, matrix, or delayed release coating. Examples of such carriers, excipients, vehicles, and diluents, can be found in Remington's Pharmaceutical Sciences, 18th Edition, A.R. Gennaro, Ed., Mack Publishing Company, Easton, PA, 1990.

Any of the compounds according to the present invention can be formulated by the skilled in the art to provide medicaments which are suitable for parenteral, buccal, rectal, vaginal, transdermal, pulmonary or oral routes of administration.

The type of formulation of the medicament containing the compound can be selected according to the desired rate of delivery. For example, if the compounds are to be rapidly delivered, the nasal or intravenous route is preferred.

The medicaments can be administered to mammals, including humans, at a therapeutically effective dose which can be easily determined by one of skill in the art and which can vary according to the specie, age, sex and weight of the treated patient or subject as well the route of administration. For example, in humans, when intravenously administered, the preferred dose falls in the range from about 1 μg to about 25 μg of total compound per kg of body weight. When orally administered, higher amounts are generally necessary. For example, in humans for the oral administration, the dosage level is typically from about 30 μg to about 1000 μg of total compound per kg of body weight. The exact level can be easily determined empirically based on the above disclosure.

EXAMPLES

The following examples illustrate the efficacy of the most preferred compounds used in the tumor treatment of this invention.

1. Materials and Methods

a) Chemicals

Hexarelin (His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2), Ala-Hexarelin (Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2), Tyr-Ala-Hexarelin (Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2), MK0677 (N-[1(R)([1,2-dihydro-1-methanesulfonylspiro-(3H-indole,3,4'-piperidin)-1'yl]-2-(phenylmethoxy)ethyl]-2-

amino-methylpropanamide-methanesulfonate), EP80317 (HAIC-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2) and D-(Lys)$_3$-GHRP6 (His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2) were supplied by Europeptides (Argenteuil, France). Human GHRH (GHRH 1-44) and somatostatin (somatostatin 1-14) were purchased from Bachem (Bubendorf, Switzerland). Human recombinant epidermal growth factor (EGF) and all tissue culture reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ^{3}H-Thymidine was purchased from Pharmacia-Amersham Italia (Milan, Italy).

b) Human tissues

Surgical tumor specimens were collected from the Department of Biomedical Sciences and Human Oncology (Division of Pathology) of the University of Turin. A tumor fragment adjacent to that used for histopathological diagnosis was immediately frozen at -80 °C and stored for 2 to 60 months until further processed for binding studies. Samples of 13 invasive breast carcinoma (10 ductal and 3 lobular), 14 non-endocrine lung carcinomas (5 squamous cell and 9 adenocarcinomas), 11 endocrine tumors of the lung, 9 endocrine tumors of the pancreas and 12 thyroid carcinomas (7 of follicular origin and 5 of medullary origin) were used. Non-neoplastic normal tissues of the corresponding organs were also analysed in parallel with the individual tumors.

c) Tumor cell lines

Human lung carcinoma cells (CaLu1), T47D and MDA-MB231, respectively, human oestrogen dependent and oestrogen

independent breast cancer cell lines were purchased from the ATCC (Rockville, MD, USA). Cells were routinely cultured in 25 cm^3 flasks at 37 °C, 5% CO_2 and 95% humidified atmosphere in RPMI supplemented with 10% FCS, penicillin-streptomycin and fungizone. When a subconfluent state was reached, cells were detached from the flasks with trypsin/EDTA.

d) GHRP receptor assay

GHRP receptors were measured on tumor membranes as described in G. Muccioli et al., Journal of Endocrinology, 157, 99-106, 1998, using ^{125}I-Tyr-Ala-Hexarelin as a ligand. Specific binding was calculated as the difference between binding in the absence and in the presence of excess unlabelled Tyr-Ala-Hexarelin and expressed as a percentage of the radioactivity added. Saturation and competition binding studies were analyzed with the GraphPAD Prism 2 program (GraphPAD Software, San Diego, CA, USA).

e) Cell proliferation studies

DNA synthesis was evaluated by ^{3}H-thymidine incorporation as described in G. Muccioli et al., Journal of Endocrinology, 153, 365-371, 1997. Starved cells were incubated with medium alone (basal) or EGF (1 ng/ml) in the absence or in the presence of different concentrations (from 10^{-8} to 10^{-6} mol/l) of Hexarelin, Ala-Hexarelin, Tyr-Ala-Hexarelin, MK0677, (D-Lys)$_3$-GHRP6 or EP80317. After incubation for 20 hours, ^{3}H-thymidine was added and incubation was continued for a further 4 hours. The reaction was halted and the cells were

harvested onto glass-fiber filter strips. Incorporation of ^{3}H-thymidine was measured in a scintillation counter.

Cell growth studies were carried out as described in P.Cassoni et al., Virchows Archiv, 425, 467-472, 1994. Cells were seeded in triplicate in 24-multiwell plates at a density of 5,000-10,000 cells/ml. Twenty-four hours after plating the medium was changed. Hexarelin or Ala-Hexarelin were added where requested at concentrations ranging from 10^{-8} to 10^{-6} mol/l. The medium was changed every 48 hours. Cells were counted at 48 and 72 or 96 hours of treatment in a double blind analysis by two independent investigators using a haemocytometer.

f) Statistical analysis

Data were expressed as means (figs. 1 and 2) or means ± S.E.M. (figs. 3 to 7) unless otherwise specified. Statistical significance was determined using Mann-Whitney test (figs. 1 to 3) or by one-way ANOVA (figs. 4 to 7). All experiments were carried out at least in triplicate.

2. Results

a) Identification of receptors for GHRP and their antagonists in different human tumors

Figure 1 shows the distribution of radiolabelled Tyr-Ala-Hexarelin binding to membranes from different endocrine and non-endocrine human tumors of various origins (*P<0.01 vs. the corresponding non-tumoral tissue). Non-endocrine tumors of the lung and breast, as well

endocrine carcinomas of the pancreas and thyroid (follicular type) showed a median specific binding value which was statistically higher than that found in the corresponding non tumoral normal tissue. In contrast, no difference in the specific binding values was observed between normal tissue and endocrine tumors of the lung or thyroid (medullary type).

b) Biochemical characteristics of receptors for GHRP and their antagonists

To determine whether the binding of ^{125}I-Tyr-Ala-Hexarelin to tumor membranes shows the properties typical of ligand-receptor interaction, the binding of radiotracer was investigated in more detail in a non-endocrine carcinoma of lung origin which displayed the highest specific binding value. Figure 2 reports the binding of ^{125}I-Tyr-Ala-Hexarelin to tumor membranes as a function of increasing concentrations of radioligand. This study revealed evidence of saturable specific binding and Scatchard analysis (upper panel) indicated the presence of a single class of high affinity sites.

The specificity of ^{125}I-Tyr-Ala-Hexarelin binding was established by determining the ability of different compounds to compete with the radioligand for the tumoral binding sites (cf. Fig. 3). The binding of radiotracer was displaced in a dose-dependent fashion by Hexarelin, Ala-Hexarelin, Tyr-Ala-Hexarelin and GHRP antagonists such as D-(Lys)$_3$-GHRP6 and EP 80317, an (Amino-azepino-indol)$_1$-D-(Lys)$_3$ derivative of Hexarelin which does not release GH in neonatal rats. A negligible displacement was observed in the presence of MK0677, a non-peptidyl

GHRP mimetic that bind to pituitary GHRP receptors. In contrast, no competition was observed in the presence of GHRH or somatostatin.

c) Effect of GHRP and their antagonists on ^{3}H-thymidine incorporation

Hexarelin at 10-6 mol/l was able to inhibit both basal and the EGF-stimulated ^{3}H-thymidine incorporation in human cells of lung carcinoma (cf. Fig. 4; *P<0.05, **P<0.01 vs. control). This antiproliferative effect was also observed when the cells were incubated in the presence of 10^{-6} mol/l Ala-Hexarelin, Tyr-Ala-Hexarelin or GHRP antagonists such as $(D\text{-}Lys)_3$-GHRP6 and EP80317. In contrast, a slight inhibition was observed in the presence of MK0677. Experiments using increasing concentrations of Hexarelin, Ala-Hexarelin, Tyr-Ala-Hexarelin, $(D\text{-}Lys)_3$-GHRP6 and EP80317 (cf. Fig. 5) revealed that these compounds inhibited the proliferative effect of EGF on human lung carcinoma cells inhibited in a dose-dependent fashion. The EC_{50} value was 5.6×10^{-8} mol/l for EP80317, 6.5×10^{-8} mol/l for Tyr-Ala-Hexarelin, 8×10^{-8} mol/l for Hexarelin, 9×10^{-8} mol/l for $(D\text{-}Lys)_3$-GHRP6 and 1×10^{-7} mol/l for Ala-Hexarelin.

d) Effect of GHRP on cell growth

In human lung carcinoma cells Hexarelin at 10^{-8} mol/l caused a decrease in cell number compared with the control with a significant effect (-47%) only after 96 hours. This effect further increased at 10^{-7} mol/l and 10

^{6} mol/l and was observed at any time point tested (cf. Fig. 6; **P<0.001; ***P<0.0001 vs. control).

In human breast cancer T47D cells Hexarelin at 10^{-8} mol/l caused a decrease in cell number compared with control with a significant effect (-54%) only after 96 hours. This effect further increased at 10^{-7} mol/l and 10^{-6} mol/l and was observed at any time point tested (cf. Fig. 7a; **P<0.001; ***P<0.0001 vs. control). A similar antiproliferative effect was also displayed by Ala-Hexarelin on these tumor cells (cf. Fig. 7b; **P<0.001; ***P<0.0001 vs. control).

In human breast cancer MDA-MB231 cells Hexarelin at 10^{-8} mol/l caused a decrease in cell number compared with control with a significant effect (-33%) only after 72 hours. This effect further increased at 10^{-7} mol/l and 10^{-6} mol/l and was observed at any time point tested (cf. Fig. 8a; *P<0.01; **P<0.001; ***P<0.0001 vs. control). A similar antiproliferative effect was also displayed by Ala-Hexarelin on these tumor cells (cf. Fig. 8b; *P<0.01; **P<0.001; ***P<0.0001 vs. control).

These results demonstrate that synthetic growth hormone releasing peptides and their antagonists inhibit the growth of human carcinoma cells in vitro. The antiproliferative effect is mediated by a specific receptor.

CLAIMS:

What is claimed is:

1. A method of treating a tumor in a mammal which method comprises administering to a mammal in need of said treatment a growth hormone releasing peptide or an antagonist thereof in an amount effective to reduce or inhibit proliferation of tumorigenic cells.

2. The method of claim 1, wherein the tumor is a lung, mammary, thyroid or pancreas tumor.

3. A method of treating a tumor in a mammal which method comprises administering to a mammal in need of said treatment a growth hormone secretagogue or an antagonist thereof in an amount effective to reduce or inhibit proliferation of tumorigenic cells.

4. The method of claim 3, wherein the tumor is a lung, mammary, thyroid or pancreas tumor.

5. A method of treating a mammal having a tumor provided with a receptor for growth hormone secretagogues which method comprises administering to a mammal in need of said treatment a growth hormone releasing peptide or an antagonist thereof in an amount effective to reduce or inhibit proliferation of tumorigenic cells.

6. The method of claim 5, wherein the tumor is a lung, mammary, thyroid or pancreas tumor.

7. A method of treating a mammal having a tumor provided with a receptor for growth hormone releasing peptides which method comprises administering to a mammal in need of said treatment a growth hormone secretagogue or an antagonist thereof in an amount effective to reduce or inhibit proliferation of tumorigenic cells.

8. The method of claim 7, wherein the tumor is a lung, mammary, thyroid or pancreas tumor.

9. A method of treating a tumor in a mammal which method comprises administering to a mammal in need of said treatment a therapeutically effective amount of a compound to reduce or inhibit proliferation of tumorigenic cells, wherein the compound is selected from the group consisting of

a) compounds of the general formula I

$$AA^1\text{-}AA^2\text{-}AA^3\text{-}AA^4\text{-Lys-R} \qquad (I)$$

in which:

AA^1 is IMA, GAB, INIP, TXM, AIB, His-D-Trp-, His-D-Mrp, Thr-D-Trp, Thr-D-Mrp, D-Thr-D-Trp, D-Thr-D-Mrp, D-Ala-D-Nal, IMA-D-Trp, IMA-D-Mrp, D-Thr-His-D-Trp, D-Thr-His-D-Mrp, Cys-Tyr-GAB, Ala-His-Trp, Ala-His-D-Mrp, Tyr-Ala-His-D-Trp, Tyr-Ala-His-D-Mrp, D-Ala-D-Trp, or D-Ala-D-Mrp;

AA^2 is Ala, D-Nal, D-Lys, D-Mrp, or Trp;

AA^3 is D-Trp, D-Nal, D-Trp, Mrp, D-Mrp, Phe, or D-Phe;

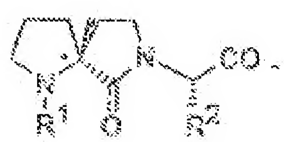
AA^4 is D-Trp, Mrp, D-Mrp, Phe, or D-Phe; and

R is $-NH_2$, Thr-NH_2, or D-Thr-NH_2; and

b) compounds of the general formula II

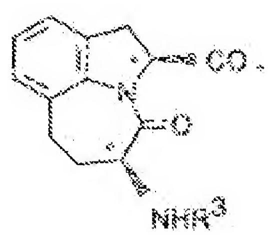
$$A - B - D\text{-Mrp} - C - E \qquad (II)$$

in which:
A is H or Tyr;
B is a spirolactam of the general formula III



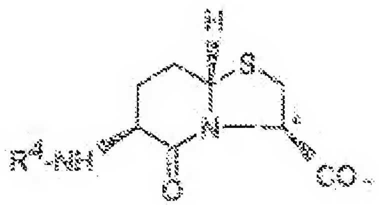
(III)

where R^1 is H or Tyr, R^2 represents the side chain of any one naturally occurring amino acid, and the configuration at * is (R), (S) or a mixture thereof; a tricyclic compound of the formula IV



(IV)

where R^3 is H or Tyr and the configuration at * is (R), (S) or a mixture thereof; a bicyclic compound of the formula V

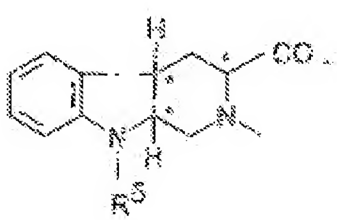


(V)

where R^4 is H or Tyr and the configuration at * is (R), (S) or a mixture thereof;

D-Mrp is Dextro-2-Methyl-Trp;

C is Trp-Phe-Lys, D-Trp-Phe-Lys, Mrp-Phe-Lys, D-Mrp-Phe-Lys, Trp-Lys, D-Trp-Lys, Mrp-Lys, D-Mrp-Lys, Ala-Trp-D-Phe-Lys, Ala-Mrp-D-Phe-Lys, Ala-D-Mrp-D-Phe-Lys, D-Lys-Trp-D-Phe-Lys, D-Lys-Mrp-D-Phe-Lys, D-Lys-D-Mrp-D-Phe-Lys, or a tricyclic compound of the formula VI



(VI)

where R^5 is H or SO_2Me and the configurations at * are either (R), (S) or a mixture thereof; and

E is Lys-NH_2 or -NH_2, provided that E is Lys-NH_2, when C is the previously defined tricyclic compound VI.

10. The method of claim 9, wherein the compound is His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2, His-D-Trp-Ala-Mrp-D-Phe-Lys-NH_2, D-Thr-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

Thr-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,
IMA-D-Mrp-D-Trp-Phe-Lys-NH_2,
IMA-D-Mrp-D-Nal-Phe-Lys-NH_2,
GAB-D-Mrp-D-Mrp-D-Mrp-Lys-NH_2,
D-Ala-D-Nal-Ala-Trp-D-Phe-Lys-NH_2,
INIP-D-Nal-D-Nal-Phe-Lys-NH_2,
INIP-D-Nal-D-Trp-Phe-Lys-NH_2,
IMA-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,
INIP-D-Mrp-D-Trp-Phe-Lys-NH_2,
INIP-D-Mrp-D-Nal-Phe-Lys-NH_2,
GAB-D-Mrp-D-Trp-Phe-Lys-NH_2,
TXM-D-Mrp-D-Trp-Phe-Lys-NH_2,
GAB-D-Mrp-Mrp-Phe-Lys-NH_2,
Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,
His-D-Mrp-Ala-Trp-D-Phe-Lys-Thr-NH_2,
His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,
D-Thr-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,
GAB-D-Mrp-D-Nal-Phe-Lys-NH_2,
GAB-D-Mrp-D-Mrp-Mrp-Lys-NH_2,
Cys-Tyr-GAB-D-Mrp-D-Mrp-Mrp-Lys-NH_2,
Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2, or
D-Ala-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2.

11. The method of claim 9, wherein the compound is
His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2,
His-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,
His-Ala-D-Trp-Lys-Mrp-D-Phe-Lys-NH_2,
His-D-Mrp-D-Lys-Mrp-D-Phe-Lys-NH_2,
His-Ala-D-Trp-Ala-Mrp-D-Phe-Lys-NH_2, or
His-D-Trp-Ala-Mrp-D-Phe-Lys-NH_2.

12. The method of claim 9, wherein the compound is
[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Trp-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-Mrp-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

Tyr-[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Ile)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-HNH-(SO_2CH_3)-Phe-Lys-NH_2,

HAIC-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2, or

ATAB-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2.

13. The method of claim 9, wherein the tumor is a lung, mammary, thyroid or pancreas tumor.

14. The method of claim 13, wherein the compound administered to the mammal displaces the radioactive marker ^{125}I-Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2 from a tumor containing tissue of said mammal.

15. The method of claim 2, wherein the compound administered to the mammal displaces the radioactive marker ^{125}I-Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2 from a tumor containing tissue of said mammal.

16. The method of claim 4, wherein the compound administered to the mammal displaces the radioactive marker ^{125}I-Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2 from a tumor containing tissue of said mammal.

17. The method of claim 6, wherein the compound administered to the mammal displaces the radioactive marker ^{125}I-Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2 from a tumor containing tissue of said mammal.

18. The method of claim 8, wherein the compound administered to the mammal displaces the radioactive marker ^{125}I-Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2 from a tumor containing tissue of said mammal.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/08082

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/08 A61K38/29 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 18016 A (DEGHENGHI ROMANO) 28 November 1991 (1991-11-28) page 5, line 15 - line 24; claims; examples	1,5,7
X	WO 95 16707 A (UNIV TULANE ;SCHALLY ANDREW VICTOR (US); ZARANDI MARTA (US)) 22 June 1995 (1995-06-22) page 9, line 13 - line 21; claims; examples	1-7
X	WO 98 44922 A (PASTERNAK ALEXANDER ;PATCHETT ARTHUR A (US); CHAPMAN KEVIN (US); Y) 15 October 1998 (1998-10-15) page 38, line 32 - page 39, line 10; claims; examples	1-7

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

Date of the actual completion of the international search

18 February 2000

Date of mailing of the international search report

22/02/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Fuhr, C

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/09562

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 807 988 A (DESHERNEY ROMANO) 15 September 1998 (1998-09-15) column 8, line 6 - column 9, line 6; examples	1,14-18
A	EP 0 395 417 A (UNIV TULANE) 31 October 1990 (1990-10-31) page 7, line 36 - line 54; claims; examples	1-7

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT	International application No. PCT/EP 99/ 05062

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 99/08682

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			DE	69016691	T	05-10-1995
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Exhibit 3: U.S. Patent No. 6,025,471 to Deghenghi

US006025471A

United States Patent [19]

Deghenghi

[11] Patent Number: 6,025,471

[45] Date of Patent: Feb. 15, 2000

[54] DIAZASPIRO, AZEPINO AND AZABICYCLO THERAPEUTIC PEPTIDES

[76] Inventor: Romano Deghenghi, Chesn-x-Dessus, St. Cergue, Switzerland, 1264

[21] Appl. No.: 09/003,884

[22] Filed: Jan. 3, 1998

[51] Int. Cl.[7] C07K 7/00

[52] U.S. Cl. 530/330; 530/329; 514/17; 540/484

[58] Field of Search 514/18, 19, 17; 530/330, 331, 329; 540/484

[56] References Cited

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R. Deghenghi, "Small Peptides as Potent Releasers of Growth Hormone", *Journal of Pediatric Endocrinology & Metabolism*, 8:311–313 (1995).

R. Deghenghi, "The development of 'impervious peptides' as growth hormone secretagogues", *Acta Paediatr. Suppl.*, 423:85–7 (1997).

Primary Examiner—Michael P. Woodward
Assistant Examiner—David Lukton
Attorney, Agent, or Firm—Pennie & Edmonds LLP

[57] ABSTRACT

The present invention relates a number of novel peptide sequences which include a spirolactam, bicyclic or tricyclic peptidomimetic unit. The peptides disclosed herein exhibit binding to cardiac tissue and normalize cardiac pressure after administration, as well as diagnostic and therapeutic properties for certain neoplastic tissues. Importantly, these peptides do not release pituitary hormones such as corticotropin (ACTH) and growth hormone (GH), and are therefore devoid of certain unwanted side-effects. These peptides preferably have at least one lysine unit and at least one D-2-alkyl-tryptophan unit.

18 Claims, 1 Drawing Sheet

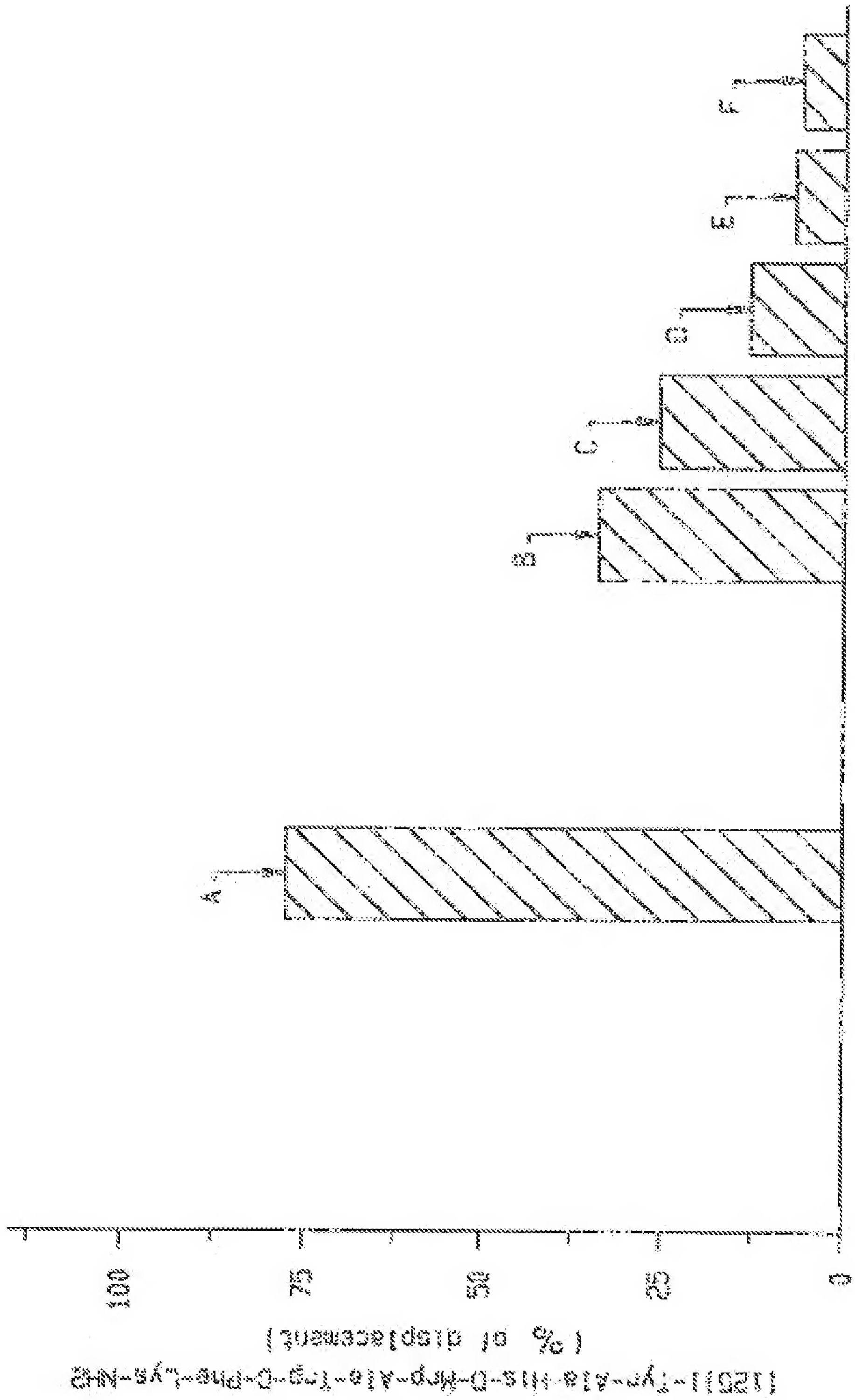


FIG. 1

DIAZASPIRO, AZEPINO AND AZABICYCLO THERAPEUTIC PEPTIDES

BACKGROUND OF THE INVENTION

The present invention relates to new peptides which include peptidomimetic units therein to stabilize and enhance their performance and bioavailability.

Under the general term heart disease, a variety of cardiac ailments, including myocardial ischemia, heart failure and related vascular dysfunction, are treated with drugs such as organic nitrates, calcium channel blockers, β-adrenergic receptor antagonists, antiplatelet and antithrombotic agents, cardiac glycosides, angiotensin converting enzyme inhibitors and angiotensin receptor antagonists. A general review of the field is found, for example, in Goodman & Gilman's "The Pharmacologic Basis of Therapeutics", IX edition, McGraw-Hill, New York, (1996), chapters 32 and 34.

Recently, the protective effect of a peptide known as Hexarelin (also called examorelin) having the structure His-D-2-methyl-Trp-Ala-Trp-D-Phe-Lys-NH_2 was described in an article by V. De Gennaro Colonna et al., European J. Pharmacology, 334, (1997), 201–207. Hexarelin was found to reverse the worsening of cardiac dysfunction in growth hormone deficient rats. At least part of its beneficial effect on myocardial ischemia was attributed to the growth hormone liberating properties of the peptide.

Heart disease is an increasing health problem as the population at large ages, such that there is a need for additional drugs or agents for treatment of these conditions. A number of the peptides of the present invention are useful for this purpose.

SUMMARY OF THE INVENTION

The present invention relates new peptides which include a spirolactam, bicyclic or tricyclic peptidomimetic unit.

Many of the peptides disclosed herein also exhibit binding to cardiac tissue and have been found to normalize cardiac pressure after administration, thus imparting cardiac protecting activity by a mechanism which at the present is unknown. One common feature for these peptides is that at least one lysine unit is present. Also, those having at least one Mrp unit are preferred for this use.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graphical illustration of the ability of certain peptides to bind to heart tissue.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In this description, the following abbreviations are used: D is the Dextro enantiomer, GH is growth hormone, Mrp is 2-Alkyl-Trp, where the Alkyl group has one to three carbon atoms, {IMA is imidazolylacetyl, GAB is γ-amino butyryl, INIP is isonipecotinyl, AIB is amino isobutyryl, Nal is β-naphthylalanine, TXM is tranexamyl (i.e., 4-(amino methyl)-cyclohexane carbonyl),} D-Hnh is D-1,2,3,4,5,6-hexahydronorharman-3-carboxylic acid, HAIC is (2S,5S)-5-amino-1,2,4,5,6,7-hexahydro-azepino[3,2,1-hi]indole-4-one-2-carboxylic acid, ATAB is 2-R-(2β,5β,8β) 8-amino-7-oxo-4-thia-1-aza-bicyclo[3.4.0]nonan-2-carboxylic acid, and Ala, Lys, Phe, Trp, His, Thr, Cys, Tyr, Leu and Ile are the amino acids Alanine, Lysine, Phenylalanine, Tryptophan, Histidine, Threonine, Cysteine, Tyrosine, Leucine and Isoleucine, respectively.

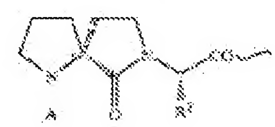
These peptides are novel and have the formula:

A-B-D-Mrp-C-E

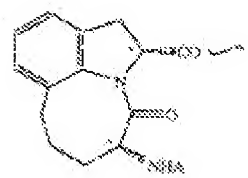
in which:

A is H or Tyr;

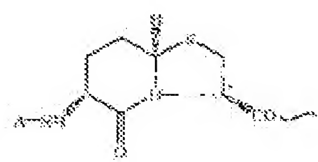
B is a spirolactam substituent of the formula



where, R^2 represents the side chain of any one naturally occurring amino acid, and the configuration at * is (R), (S) or a mixture thereof; a tricyclic substituent of the formula:



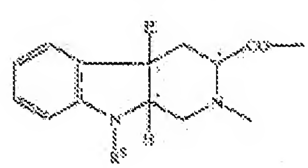
where the configuration at * is (S), (R) or a mixture thereof; a bicyclic substituent of the formula:



where the configuration at * is (R), (S) or a mixture thereof;

D-Mrp is Dextro-2-Alkyl-Trp, where the Alkyl group contains 1 to 3 carbon atoms and is preferably methyl;

C is Trp-Phe-Lys, D-Trp-Phe-Lys, Mrp-Phe-Lys, D-Mrp-Phe-Lys, Trp-Lys, D-Trp-Lys, Mrp-Lys, D-Mrp-Lys, Ala-Trp-D-Phe-Lys, Ala-Mrp-D-Phe-Lys, Ala-D-Mrp-D-Phe-Lys, D-Lys-Trp-D-Phe-Lys, D-Lys-Mrp-D-Phe-Lys, D-Lys-D-Mrp-D-Phe-Lys, or a tricyclic substituent of the formula:



where R^5 is H or SO_2Me and the configurations at * are either (R), (S), or a mixture thereof; and preferably

E is Lys-NH_2 or —NH_2, provided that E is preferably Lys-NH_2 when C is the previously defined tricyclic substituent.

The preferred novel peptidomimetic containing peptides include the following:

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Trp-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-Mrp-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

Tyr-[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-NH_2,

Tyr-[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

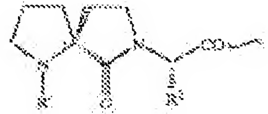
[S,S-Spiro(Pro-Ile)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Hnb(SO_2CH_3)-Lys-NH_2,

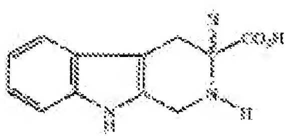
HAIC-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2, and

ATAB-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

where S,S-Spiro(Pro-Leu) and S,S-Spiro(Pro-Ile) is 4-Methyl-2S[6'-oxo(5'-S)1',7'-diazaspiro[4,4]nonan-7'-yl-]pentanoic acid. These substituents have the formula



where R^2 is the side chain of Leu or Ile (see P. Ward et al., *J. Med Chem*, 33, 1848 (1990)). Also, the tricyclic compound Hnb is obtained by conventional hydrogenation of the corresponding tetrahydronorharman-3-carboxylic acids of the formula:



The peptidomimetic units which are advantageous for use in the peptides of the invention include those which are locking in a β-turn configuration which mimic the natural amino acids. The spirolactam, bicyclic and tricyclic substituents defined above are preferred.

Pharmaceutically acceptable salts of the peptides of the present invention include can be used, if desired. Such salts would include organic or inorganic addition salts, including hydrochloride, hydrobromide, phosphate, sulfate, acetate, succinate, ascorbate, tartrate, gluconate, benzoate, malate, fumarate, stearate and pamoate salts. They can also be administered in controlled release formulations such as subcutaneous implants or intramuscular microcapsules and the like.

All these peptides can be conveniently synthesized according to the usual methods of peptide chemistry, such as by solid phase peptide synthesis, as described by E. Atherton and R. C. Sheppard in "Solid Phase Peptide Synthesis" IRL Press at Oxford University Press 1989, by solution phase synthesis as described by J. Jones in "The Chemical Synthesis of Peptides", Clarendon Press, Oxford 1994, or by both solid- and solution-phase methods, as known in the art.

The solid-phase synthesis starts from the C-terminal end of peptide. A suitable starting material can be prepared, for example, by attaching the required protected alpha-amino acid to a chloromethylated resin, a hydroxymethylated resin, a benzhydrylamine resin (BHA), or to a paramethylbenzhydrylamine resin (p-Me-BHA). As an example, an available chloromethylated resin is BIOBEADS® SX 1 by BioRad Laboratories, Richmond, Calif. The preparation of the hydroxymethyl resin is described by Bodansky et al., Chem. Ind. (London) 38, 15997 (1966). The BHA resin is described by Pietta and Marshall, Chem. Comm., 650 (1970) and is commercially available by Peninsula Laboratories Inc., Belmont, Calif.

After the starting attachment, the protecting group of the alpha-amino acid can be removed by means of different acid reagents, comprising trifluoroacetic acid (TFA) or hydrochloric acid (HCl) dissolved in organic solvents at room temperature. After the removal of the protecting group of the alpha amino acid, the remaining protected amino acids can be coupled step by step in the desired order. Each protected amino acid can generally be reacted in excess of about three times using a suitable carboxyl activating group, such as dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC) dissolved, for example, in methylene chloride (CH_2Cl_2), dimethylformamide (DMF) or their mixtures. After the desired aminoacidic sequence has been completed, the desired peptide can be cleaved from the supporting resin by treatment with a reagent such as hydrogen fluoride (HF) which cleaves not only the peptide from the resin, but also the protecting groups of the lateral chains. When a chloromethylated resin or a hydroxymethylated resin is used, the treatment with HF leads to the formation of the terminal acid peptide in free form. When a BHA or p-Me-BHA resin is used, treatment with HF directly leads to the formation of the terminal amide peptide in free form.

Medicaments of these peptides can be administered to an animal, preferably a mammal and including a human. These medicaments can comprise a peptide of the present invention or a pharmaceutically acceptable salt thereof, or combinations of peptides of the present invention or pharmaceutically acceptable salts thereof, optionally, in admixture with a carrier, excipient, vehicle, diluent, matrix or delayed release coating. Examples of such carriers, excipients, vehicles and diluents, can be found in *Remington's Pharmaceutical Sciences*, 18th Edition, A. R. Gennaro, Ed., Mack Publishing Company, Easton, Pa., 1990.

These medicaments can be administered to animals, including humans, at a therapeutically effective dose which can be easily determined by one of skill in the art and which can vary according to the specie, age, sex and weight of the treated patient or subject. For example, in humans, when intravenously administered, the preferred dose falls in the range from about 1 μg to about 25 μg of total peptide per kg of body weight. When orally administered, typically higher amounts are necessary. For example, in humans for the oral administration, the dosage level is typically from about 30 μg to about 1000 μg of polypeptide per kg of body weight. The exact level can be easily determined empirically based on the above disclosure.

Any of the peptides of the present invention can be formulated by the skilled in the art to provide medicaments which are suitable for parenteral, buccal, rectal, vaginal, transdermal, pulmonary or oral routes by adjusting the dose as needed, such doses being in the range of from about 1 μg/kg to 1 mg/kg of body weight as noted above depending on the rate of absorption and the potency of the peptide.

These peptides possess useful therapeutic properties. In particular, many have cardioprotectant and in general beneficial cardiovascular properties. In addition, some have diagnostic and therapeutic properties for certain neoplastic tissues. Importantly, these peptides do not release pituitary hormones such as corticotropin (ACTH) and growth hor-

mone (GH), and are therefore devoid of certain unwanted side-effects. For diagnostic purposes, the radioactive iodine derivatives on the initial tyrosine are particularly useful.

EXAMPLES

Example 1

Data is presented for the most preferred lysine containing peptides of the invention. The GH releasing effect was measured in rats according to the method described by R. Deghenghi et al., *Life Sci.* 54: 1321–1328 (1994). The cardiac protection of the instant peptides has been measured essentially as described in the publication by V. De Gennaro Colonna et al., *Europ. J. Pharmacol.* 334:201–207 (1997).

The binding abilities of certain peptides according to the invention compared to conventional peptides on human heart membranes are shown in FIG. 1. These data have been obtained according to the method of G. Muccioli et. al., *J. Endocrinology*, 156, 99 (1998). Data for the peptides used are shown in the graph using the following identifications.

no.	peptide
A	[Spiro (S,S)-(Pro—Leu)]-D-Mrp-D-Trp—Phe—Lys—NH_2
B	D-Mrp-D-Mrp—Phe—NH_2
C	GAB-D-Mrp-D-Mrp—NH_2
D	D-Mrp—Mrp—NH_2
E	AIB-D-Mrp—Mrp—NH_2
F	AIB-D-Mrp-D-Mrp—NH_2

Peptide A is in accordance with the invention, while peptides B-F are comparative. As shown in the figure, peptide A provided inhibition (i.e., displacement) of [125]I-Tyr-Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2 in a proportion of about 75%, whereas peptides B-F only provided about 5 to less than 35%. The greater binding affinities for the peptides of the invention illustrate that these peptides directly operate on specific receptors of heart tissue to achieve normalization of cardiac pressure.

Example 2

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Trp-Phe-Lys-NH_2. By conventional solid phase synthesis, the title peptide was obtained and purified as the acetate salt. Theoretical molecular weight 915.2 +1; Found 915.5

Example 3

[S,S-Spiro(Pro-Leu)]-D-Mrp-Mrp-Lys-NH_2. Following the procedure of Example 2, the title peptide was similarly obtained as the acetate salt. Theoretical M W. 782; Found 781.7

Example 4

[S,S-Spiro(Pro-Leu)]-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2. The title compound was prepared in a similar procedure as in Example 2 and purified as the acetate salt. Theoretical M.W. 986.2 ; Found 986.2

Example 5

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2. Similarly to Example 2, the title peptide was obtained as the acetate salt. Theoretical M.W. 1043.2; Found 1042.9

Example 6

Tyr-[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2. Similarly to Example 5, the title peptide was obtained as the acetate salt. Theoretical M.W. 1206.5; Found 1206.3

Example 7

As in Example 4, by a similar procedure, the title compound was obtaiu ed as the acetate salt. Theoretical M. W. 1043.3; Found 1043.0

Example 8

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Hnb(SO_2CH_3)-NH_2. By a solution phase method, the title compound was obtained as the acetate salt. Theoretical M.W. 731.9; Found 732.4

Example 9

Halo-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2. Similarly to Example 7, the title peptide was obtained as the acetate salt. Theoretical M.W. 1027.3; Found 1027.0

Example 10

Aib-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2. Following a procedure similar to Example 8, the title compound was obtained as the acetate salt. Theoretical M.W. 1005.3; Found 1005.0

Example 11

The conversion of water soluble salts of any peptide described in Examples 2 to 10 above into water insoluble salts (e.g. pamoates or stearates) is obtained by treating an aqueous solution of the water soluble salts with the equivalent amount of an aqueous solution of sodium pamoate, or sodium stearate, and filtering the insoluble peptide salt which precipitates out of the solution. The dried insoluble salt can be used without further purification.

Examples 12–14

These examples illustrate preferred formulations for administration of the peptides of the invention.

Example 12

The peptide of Example 2 is lyophilized in sterile vials containing 100 micrograms of the peptide and 10 mg of mannitol as excipient. Water for injection is then used to dissolve the peptide into a formulation which can be injected i.v. into mammals with impaired cardiac function at a dose of 1 μg/kg body weight.

Example 13

The peptide of Example 3 is compounded with mannitol in a dry state (1:10) and then filled into soft gelatin capsules at a dose of 20 mg peptide (200 mg mannitol). The resulting capsule can be administered orally to mammals experiencing cardiac failure.

Example 14

The peptides of Examples 4 and 5 are dissolved in sterile water containing 0.05% of chlorocresol as a preservative. This solution can be administered intranasally at doses of 20 to 60 μg/kg twice or three times daily to mammals with impaired heart function so that the peptides can be rapidly absorbed.

What is claimed is:

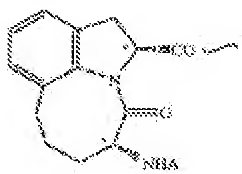
1. A peptide of the formula:

A-B-D-Mrp-C-E

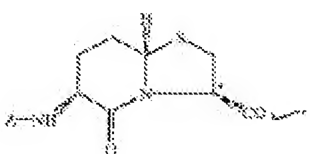
in which:

B is a spirolactam substituent of the formula

where A is H or Tyr, R^2 represents the side chain of any one naturally occurring amino acid, and the configuration at * is (R), (S) or a mixture thereof; a tricyclic substituent of the formula:

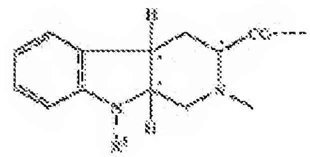


where A is H or Tyr and the configuration at * is (S), (R) or a mixture thereof; a bicyclic substituent of the formula:



where A is H or Tyr and the configuration at * is (R), (S) or a mixture thereof; D-Mrp is Dextro-2-Alkyl-Trp, where the Alkyl group contains 1 to 3 carbon atoms;

C is Trp-Phe, D-Trp-Phe, Mrp-Phe, D-Mrp-Phe, Trp, D-Trp, Mrp, D-Mrp, Ala-Trp-D-Phe, Ala-Mrp-D-Phe, Ala-D-Mrp-D-Phe, D-Lys-Trp-D-Phe, D-Lys-Mrp-D-Phe, D-Lys-D-Mrp-D-Phe, or a tricyclic substituent of the formula:



where R^5 is H or SO_2Me and the configurations at * are either (R), (S), or a mixture thereof; and

E is Lys-NH_2 or -NH_2.

2. The peptide of claim 1 that contains a spirolactam substituent where R^2 is the side chain of Leu or Ile.

3. The peptide of claim 1 that contains a Lys unit.

4. The peptide of claim 1 that contains a D-Mrp unit.

5. The peptide of claim 1 specifically as

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Trp-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-Mrp-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

Tyr-[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-NH_2,

Tyr-[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

[S,S-Spiro(Pro-Leu)]-D-Mrp-D-Hni(SO_2CH_3)-Lys-NH_2,

[S,S-Spiro (Pro-Ile)]-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2,

HAIC-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2, or

ATAB-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH_2.

6. A pharmaceutical formulation suitable for parenteral use containing a peptide of claim 1 and a suitable carrier.

7. The pharmaceutical formulation of claim 6 wherein the peptide is present as a pharmaceutically acceptable water soluble salt.

8. The pharmaceutical formulation of claim 6 wherein the peptide is present as a pharmaceutically acceptable water insoluble salt.

9. The pharmaceutical formulation of claim 6 wherein the peptide is present in a matrix of a biodegradable material.

10. The pharmaceutical formulation of claim 6 wherein the peptide is present in an amount of 1 mg to 1 mg/kg per body weight of a mammal to which it is to be administered.

* * * * *

Exhibit 4: Bodart *et al.*, *Circ. Res.* 90:844-849 (2002)

CD36 Mediates the Cardiovascular Action of Growth Hormone–Releasing Peptides in the Heart

V. Bodart, M. Febbraio, A. Demers, N. McNicoll, P. Pohankova, A. Perreault, T. Sejlitz, E. Escher, R.L. Silverstein, D. Lamontagne, H. Ong

Abstract—Growth hormone–releasing peptides (GHRPs) are known as potent growth hormone secretagogues whose actions are mediated by the ghrelin receptor, a G protein–coupled receptor cloned from pituitary libraries. Hexarelin, a hexapeptide of the GHRP family, has reported cardiovascular activity. To identify the molecular target mediating this activity, rat cardiac membranes were labeled with a radioactive photoactivatable derivative of hexarelin and purified using lectin affinity chromatography and preparative gel electrophoresis. A binding protein of M_r 84 000 was identified. The N-terminal sequence determination of the deglycosylated protein was identical to rat CD36, a multifunctional glycoprotein, which was expressed in cardiomyocytes and microvascular endothelial cells. Activation of CD36 in perfused hearts by hexarelin was shown to elicit an increase in coronary perfusion pressure in a dose-dependent manner. This effect was lacking in hearts from CD36-null mice and hearts from spontaneous hypertensive rats genetically deficient in CD36. The coronary vasoconstrictive response correlated with expression of CD36 as assessed by immunoblotting and covalent binding with hexarelin. These data suggest that CD36 may mediate the coronary vasospasm seen in hypercholesterolemia and atherosclerosis. (*Circ Res.* 2002;90:844-849.)

Key Words: acute coronary syndromes ■ growth hormone–releasing peptides ■ CD36 scavenger receptor

Growth hormone–releasing peptides (GHRPs) belong to a family of small synthetic peptides modeled from Met-enkephalin, which exhibit potent and dose-dependent GH-releasing activity and also significant prolactin (PRL)- and corticotropin (ACTH)-releasing effects.[1] These neuroendocrine activities of GHRPs are mediated by the Ghrelin receptor, a specific G protein–coupled receptor[2,3] that has been cloned from mammalian pituitary libraries and its subtypes identified in the pituitary gland, hypothalamus, and extra-hypothalamic brain regions by binding studies.[4] Equilibrium displacement binding assays with GHRPs in different peripheral tissues have shown specific binding sites in the heart, adrenal, ovary, testis, lung, and skeletal muscle.[5,6] Significantly, hexarelin, a hexapeptide member of the GHRPs family has been reported to feature cardiovascular activity. Long-term pretreatment of GH-deficient rats with this peptide provided protective effect on hearts from ischemia/reperfusion damages[7] and prevented alterations of the vascular endothelium-dependent relaxant function.[8] This protective effect was independent of any stimulation of the somatotropic axis,[8,9] suggesting a direct action of hexarelin on specific cardiac receptors. Our initial characterization of a putative cardiac GHRP receptor revealed the existence of a binding site for a photoactivatable derivative of hexarelin with a M_r of 84 000 distinct from those identified in the pituitary.[6,10] In the present study, we report the identification of the unique GHRP binding site in the heart as CD36, a multifunctional B-type scavenger receptor. We also demonstrate that the activation of this receptor by hexarelin induced a dose-dependent increase in coronary perfusion pressure in isolated perfused hearts. This effect was absent in hearts from CD36-deficient animals. These studies demonstrate a novel function for this scavenger receptor in the regulation of the vascular tone and suggest a potential role for CD36 in pathological vasospasm.

Materials and Methods

Animals

Hearts from male Sprague-Dawley rats (>400 g, n=110; Charles River, St Constant, Quebec, Canada) were used as source of cardiac membranes for the purification of the hexarelin binding protein. Langendorff perfused heart experiments were performed on spontaneously hypertensive rats/NCrlBR (SHR/NCrlBR) (n=5) and their control strain Wistar-Kyoto/NCrlBR (WKY/NCrlBR) (300 to 325 g, n=5 Charles River) as well as on CD36-null mice (n=8) and their control strain C57Bl/6[11] (n=8).

Membrane Preparation

Animal use was in accordance with the Canadian council on animal care guidelines. All animals were anesthetized with sodium pento-

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From the Faculty of Pharmacy and Department of Pharmacology (V.B., N.M., P.P., D.L., A.D., A.P. H.O.), Université de Montréal, Montreal, Canada; the Division of Hematology and Medical Oncology, Department of Medicine (M.F., R.L.S.), Weill Medical College, Cornell University, New York, NY; Biovitrum AB (T.S.), Stockholm, Sweden; and the Department of Pharmacology (E.E.), Université de Sherbrooke, Canada.

Correspondence to Dr Huy Ong, Faculty of Pharmacy, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montreal, Quebec, H3C 3J7 Canada. E-mail huy.ong@umontreal.ca

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barbital (Somnotol, 3 mg/100 g, IP) and their hearts were promptly removed and placed in ice-cold saline buffer. Cardiac membranes were prepared according to Harigaya and Schwartz.[12]

Receptor Binding and Photolabeling With [^{125}I]-Tyr-Bpa-Ala-Hexarelin

The radioiodination procedure of the photoactivatable ligand and the receptor binding assays were performed as described by Ong et al.[13] Nonspecific binding was defined as that not displaced by 10 μmol/L hexarelin.

Solubilization of Photolabeled Cardiac Membranes

Photolabeled cardiac membranes were solubilized in buffer A (50 mmol/L Tris-HCl pH 7.4, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 2 mmol/L $MnCl_2$, 1% Triton X-100, 1 μmol/L pepstatin, 1 μmol/L leupeptin, 0.1 μmol/L aprotinin, 0.4 mmol/L Pefabloc) for 20 hours at 4°C. The soluble fraction was obtained by centrifugation at 35 000*g* for 60 minutes at 4°C.

Purification of Labeled Protein and N-Terminal Sequencing

The solubilized cardiac membranes were consecutively incubated with wheat germ-agarose and lentil-Sepharose for 20 hours at 4°C. The lectin-coupled resins were washed with buffer A used in the solubilization step and the retained proteins were eluted with 0.3 mol/L *N*-acetylglucosamine and 0.5 mol/L α-methyl-D-mannopyranoside, respectively. After reduction with 5 mmol/L DTT and alkylation with 10 mmol/L iodoacetamide, the eluted proteins purified by lectin affinity chromatography were separated on 5% preparative SDS-PAGE. The radioactive band at 80 to 90 kDa was cut out of the gel and eluted in buffer B (100 mmol/L NH_4HCO_3, 0.1% SDS buffer). After acetone precipitation, the sample was reconstituted in buffer C (100 mol/L NaH_2PO_4 pH 7.0, 10 mmol/L EDTA, 10 mmol/L β-mercaptoethanol, 0.1% SDS, 0.6% octylglucoside), deglycosylated with 50 U of *N*-glycosidase F for 20 hours at room temperature, and repurified on 7.5% SDS-PAGE. The radioactive band at M_r 57 000 corresponding to the deglycosylated binding protein of hexarelin was eluted in buffer B, and an aliquot sequenced by Edman degradation using an Hewlett-Packard G1000A protein sequencer in order to obtain the N-terminal sequence of the protein.

Western Blot

Cardiac membrane proteins were quantified by the bicinchoninic acid method, electrophoresed, and transferred to nitrocellulose membrane. CD36 was detected by a polyclonal rabbit anti-rat CD36 antibody generated in our laboratory by using the peptide CD36 (164 to 183) coupled to keyhole limpet hemocyanin as immunogen. The specific anti-CD36 immunoglobulins were purified by affinity on 6% crosslinked agarose coupled to the CD36 (164 to 183) peptide. The CD36/antibody complex was visualized with a peroxidase-linked goat anti-rabbit antibody and chemiluminescent enhancement.

Recombinant Soluble CD36 Expression, Photolabeling, and Immunoprecipitation

Extracellular (152 to 1389) CD36 cDNA was cloned by reverse transcription of rat heart ventricle followed by PCR amplification of the cDNA by using AvanTaq DNA polymerase (Clonetech). Oligonucleotide primers were designed against rat adipocytes CD36 nucleotide sequence[14] in which the forward primer 5'-GAATTCCATATGCCGGTTGGAGACCTAC-3' and the reverse primer 5'-CAGGCGAATTCACTTTATTTTCCCGGTCAC-3' contained *Nde*I and *Eco*RI endonuclease restriction sites, respectively. The resulting cDNA was subcloned into pET17b vector (Novagen). The construction was transformed into *Escherichia coli* JM109. Positive recombinant plasmid rCD36-pET17b selected by ampicillin resistance was transformed into *E coli* BL21. The selected clones were subjected to induction of protein expression with IPTG 0.4 mmol/L for 2 hours at 37°C. *E coli* cells were harvested, washed, and resuspended in Tris HCl pH 8.0 (50 mmol/L) containing EDTA (5 mmol/L) and proteinase inhibitors (in μmol/L) pepstatin 1.0, leupeptin 1.0, aprotinin 0.1, and Pefabloc 0.4. Cell lysis was performed by repeated cycles of freezing and thawing and sonication. The cell lysate was then centrifuged at 14 000*g* for 10 minutes, and the supernatant containing the recombinant soluble CD36 protein was submitted to photoaffinity labeling with the radiolabeled photoactivatable hexarelin derivative as described above. After the photolabeling step, the supernatant was first precleared by immunoprecipitation with addition of preimmune rabbit serum (30 μL) and protein A agarose (60 μL) (Roche Mannheim, Germany). The photolabeled protein was then immunoprecipitated using polyclonal rabbit anti-rat CD36 antibody (30 μL) and protein A agarose (60 μL). Both immunoprecipitates bound to protein A were washed and boiled with Tris HCl buffer pH 6.8 (62.5 mmol/L) containing 2% SDS, 10% glycerol, 5% 2β-mercaptoethanol, and 0.00125% bromophenol blue. The eluted radiolabeled material were resolved on SDS-PAGE for autoradiography. *E coli* containing only the pET17b vector were processed as described above as negative control.

Experimental Protocol With Langendorff Perfused Hearts

Animal use was in accordance with the Canadian council on animal care guidelines. Rats (300 to 350 g) and mice (25 to 30 g) were narcotized with CO_2 until complete loss of consciousness and promptly decapitated. Hearts were rapidly immersed into ice-cold Krebs-Henseleit buffer, mounted within 2 minutes on the Langendorff apparatus, and perfused at a constant flow rate by means of a digital peristaltic pump as previously described.[15] The normal perfusion solution consisted of a modified Krebs-Henseleit buffer containing (in mmol/L): NaCl 118.0, KCl 4.0, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0, $NaHCO_3$ 24.0, D-glucose 5.0, and pyruvate 2.0, gassed with 95% O_2/5% CO_2 (pH 7.4), and kept at a constant temperature of 37°C. The perfusion flow rate was between 12 to 15 mL min^{-1} in rat hearts (yielding a coronary perfusion pressure of 75 mm Hg) and was set at 3 mL min^{-1} for mouse hearts. Isovolumetric left ventricular pressure, its first derivative (dP/dt), and heart rate were all measured from a fluid-filled latex balloon inserted into the left ventricle and connected to a pressure transducer. The volume of the balloon was adjusted to obtain a diastolic pressure around 10 mm Hg. Coronary perfusion pressure was recorded with a second pressure transducer connected to a side-port of the perfusion line. All these cardiac functional variables were recorded on a polygraph system (Grass Model 79 polygraph, AstroMed Inc). After a 20-minute stabilization period, dose-response curves to hexarelin were charted by successive infusions of increasing concentrations of the peptide administered through a Y connector of the aortic cannula with a syringe pump. Each infusion was maintained for 5 to 10 minutes, enough to reach steady state.

Results

Affinity Purification of GHRP Receptor in Cardiac Membranes

In our previous study,[9] the cardiac binding sites for hexarelin were identified as a heavily glycosylated membrane-associated protein. Lectin affinity chromatography was thus used as initial purification step. Among the various lectins tested, wheat germ agglutinin and lens culinaris were found to give the highest yield (30%). Solubilized photolabeled rat cardiac membranes were successively applied on wheat germ agglutinin and lens culinaris affinity columns. Figure 1, lane 2, depicts the enriched GHRP receptor fraction obtained in the eluate. This was further purified on semipreparative SDS-PAGE and the band of M_r 84 000 (Figure 1, lane 3) was eluted and treated with *N*-glycosidase F and reapplied on SDS-PAGE. The deglycosylated protein of M_r 57 000 (Figure 1, lane 4) was eluted from the gel and submitted to N-terminal

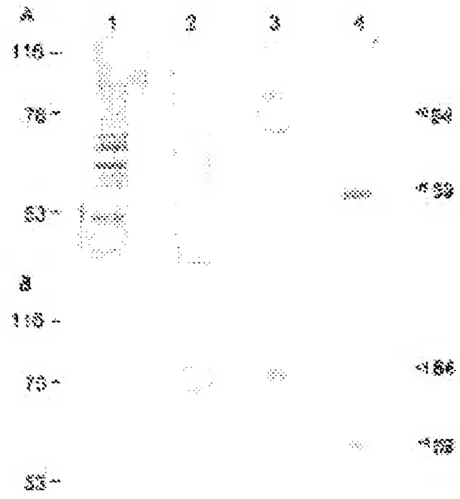

Figure 1. SDS-PAGE analysis of the successive steps of purification of the binding site of GHRP from rat heart. A, Coomassie blue staining of the gel; B, autoradiogram of the gel. Lane 1, Soluble fraction in Triton X-100 of the photolabeled cardiac membranes. Lane 2, Eluate from the lectin affinity chromatography. Lane 3, Purified fraction after the semipreparative SDS-PAGE step. Lane 4, Soluble fraction containing the deglycosylated photolabeled GHRP receptor.

sequence analysis by Edman degradation. The amino acid sequence obtained was GCDRNXGLITGAVIGAVLAFGGILMPVV, which was found identical to the N-terminal sequence of rat CD36 antigen.[15,16]

CD36 Photolabeling and Immunoblotting in SHR and CD36-Null Mice

To further demonstrate that CD36 is the binding site for GHRP in the heart, we performed photolabeling studies of cardiac membrane preparations isolated from 2 different models of CD36 deficiency: CD36-null mice by homologous recombination and rats from the SHR/NCrlBR strain. These rats have been shown to have a defective CD36 gene resulting in the generation of multiple splice variants of CD36 cDNA, with the corresponding proteins being undetectable in the plasma membrane of their adipocytes.[17] Covalent photolabeling of cardiac membranes derived from CD36-deficient rats and CD36-null mice with [^{125}I]-Tyr-Bpa-Ala-Hexarelin did not feature any specific binding signal, compared with those from control strains WKY/NCrlBR and C57Bl/6, which showed a specific photolabeled band of M_r 84 000 (Figure 2).

Figure 2. Covalent photolabeling of cardiac membranes with [^{125}I]-Tyr-Bpa-Ala-Hexarelin in the absence (−) or presence (+) of an excess of hexarelin (10 μmol/L). A, Membranes from the SHR/NCrlBR and WKY/NCrlBR strains. B, Membranes from CD36-null mice (−/−) and their wild-type littermates (+/+).

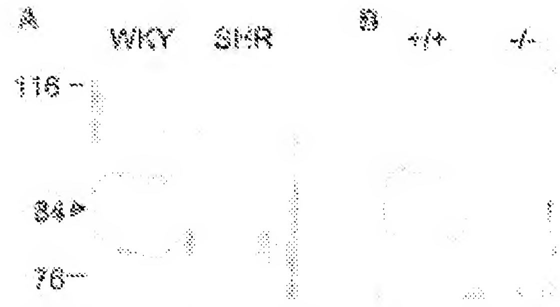

Figure 3. Immunodetection of CD36 in cardiac membranes. A, Membranes from the SHR/NCrlBR and WKY/NCrlBR strains. B, Membranes from CD36-null mice (−/−) and their wild-type littermates (+/+).

Western blot analysis of cardiac membrane proteins from SHR/NCrlBR and CD36 knockout mice using a polyclonal rabbit anti-rat CD36 antibody showed no expression of CD36, which contrasted with the high level of CD36 protein immunodetected at M_r 84 000 in cardiac membranes from WKY/NCrlBR and C57Bl/6 control strains (Figure 3). Taken together, the data of photolabeling and Western blot analysis support the evidence of a unique binding protein for hexarelin corresponding to CD36 in the heart.

Identification of CD36 as Binding Site of Hexarelin

To confirm the identity of CD36 as the interacting protein of [^{125}I]-Tyr-Bpa-Ala-Hexarelin derivative, we have expressed the extracellular binding domain of this scavenger receptor using *E coli* BL21 as vector. The photoaffinity labeling of the nonglycosylated soluble form of CD36 was carried out as described above. The immunoprecipitated material using the polyclonal rabbit anti-rat CD36 antibody, resolved by SDS-PAGE, featured a unique radioactive band at M_r 51 000 as shown in the autoradiogram (Figure 4). This band was not observed from the immunoprecipitated material using the nonimmune rabbit serum. The immunoprecipitation of the photoaffinity cross-linking of [^{125}I]-Tyr-Bpa-Ala-Hexarelin to the soluble form of CD36 generated a radiolabeled band

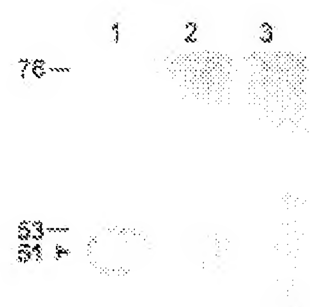

Figure 4. Immunoprecipitation of soluble CD36 recombinant protein photolabeled with [^{125}I] Tyr-Bpa-Ala-Hexarelin. Lane 1, Immunoprecipitation with polyclonal rabbit anti-rat CD36 antibody. Lane 2, Immunoprecipitation with nonimmune rabbit serum from the preclearing step. Lane 3, Immunoprecipitation with polyclonal rabbit anti-rat CD36 antibody of the lysate of *E coli* transfected with pET17b vector only (negative control).

TABLE 1. Basal Functional Variable Values and Values Under Maximal Stimulation With Either Hexarelin or Angiotensin II in Hearts From SHR and WKY

	WKY	SHR	*P*
Hexarelin			
Heart mass, g	1.68±0.04	1.81±0.92	0.30
Heart rate, min^{-1}			
Basal	237±9	221±10	0.30
Maximal	238±5	223±13	0.43
Maximum dP/dt, mm Hg s^{-1}			
Basal	2320±174	2070±124	0.28
Maximal	1840±180	1381±168	0.56
Coronary resistance, mm Hg min mL^{-1}			
Basal	5.07±0.20	6.08±0.15	0.004†
Maximal	9.68±0.60*	7.22±0.32*	0.011
Angiotensin II			
Heart mass, g	1.77±0.17	1.48±0.08	0.17
Heart rate, min^{-1}			
Basal	269±13	297±20	0.38
Maximal	274±18	290±19	0.42
Maximum dP/dt, mm Hg s^{-1}			
Basal	2980±102	2706±201	0.42
Maximal	2783±144	2262±160*	0.04
Coronary resistance, mm Hg min mL^{-1}			
Basal	5.41±0.44	8.38±0.41	0.13†
Maximal	8.56±0.61*	10.52±1.90*	0.36

Values are mean±SEM. SHR indicates spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; and *P*, probability of SHR being different from WKY, obtained with a 2-sample unpaired *t* test with separate variance (n=6 to 7 hearts per strain).

*$P<0.05$ compared with the corresponding basal value (paired *t* test); †$P<0.01$ when hearts treated with hexarelin and angiotensin II are pooled.

migrating at M_r 51 000, corresponding to the expected mass of the radioligand-nonglycosylated extracellular CD36 conjugate.

GHRP-Induced Coronary Vasoconstriction Is Mediated by CD36

We have previously reported the vasoconstrictive effect of hexarelin in the perfused rat heart model.[5] To assess whether this coronary vasoconstriction was mediated by CD36, dose-response curves to hexarelin were performed in the perfused Langendorff hearts collected from SHR/NCrIBR, CD36-null mice, and their control strains WKY/NCrIBR and C57Bl/6, respectively. The basal functional variables in hearts isolated from SHR/NCrIBR were comparable with those from WKY/NCrIBR, with the exception of coronary resistance, which was higher in the former strain (Table 1). Figure 5 (left panel) depicts the increase in coronary perfusion pressure induced by increasing concentrations of hexarelin in hearts isolated from inbreed SHR/NCrIBR and from inbreed controls (WKY/NCrIBR). The increase in coronary perfusion pressure observed at high concentrations of hexarelin in hearts from

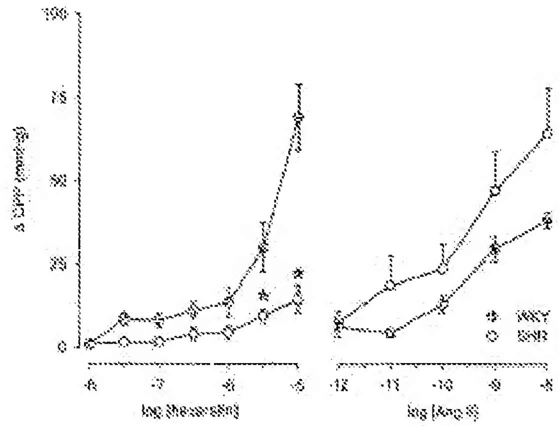

Figure 5. Change in coronary perfusion pressure (CPP) induced by increasing concentrations of hexarelin (left) and angiotensin II (Ang II, right) in hearts from SHR/NCrIBR (open circles, n=6) and WKY/NCrIBR (filled circles, n=6). *Concentrations for which a significant ($P<0.05$) difference was found between groups (analysis of variance).

WKY/NCrIBR was markedly blunted in hearts from CD36-deficient rats. Hexarelin had no chronotropic or inotropic effects in rat hearts (Table 1). The potent vasoconstrictor angiotensin II induced comparable response in hearts isolated from both strains (Figure 5, right panel), suggesting that the blunted coronary response to hexarelin from SHR/NCrIBR was not due to nonspecific effects of the elevated blood pressure in these animals.

CD36-null mice were used as a second model of CD36 deficiency. These animals had normal hearts, as shown by the comparable functional variable values between CD36-null and C57Bl/6 control mice (Table 2). A lower resting coronary resistance was observed in CD36-null mice, which was statistically significant only in the first series of experiments. Hexarelin induced a dose-dependent increase in coronary perfusion pressure in hearts from C57Bl/6 mice that was totally absent in hearts lacking the CD36 protein (Figure 6, left panel). In comparison, angiotensin II induced a dose-dependent vasoconstriction statistically comparable in hearts from both strains of mice (Figure 6, right panel). Hexarelin also induced negative chronotropic (statistically significant in C57Bl/6 mice only) and inotropic effects in mouse hearts (Table 2).

Discussion

Growth hormone (GH) secretion is well known to be regulated by GH-releasing hormone (GHRH) and somatostatin at the hypothalamic level. The discovery of growth hormone–releasing peptides has revealed the existence of a third pathway for the modulation of GH release.[18] This action on GH release is mediated by a G protein–coupled receptor of M_r 41 000, which is mainly expressed at the hypothalamic and pituitary levels.[4] Besides this neuroendocrine effect of GHRPs, it was reported that a long-term treatment with hexarelin, a hexapeptide member of the GHRP family, featured a protective effect against postischemic dysfunction in rats. Because no apparent stimulation of the growth

TABLE 2. Basal Functional Variable Values and Values Under Maximal Stimulation With Either Hexarelin or Angiotensin II in Hearts From CD36 Knockout and Control Mice

	C57BL/6	CD36−/−	P
Hexarelin			
Heart mass, mg	161±7	158±6	0.78
Heart rate, min^{-1}			
Basal	343±10	332±19	0.61
Maximal	319±13*	301±35	0.64
Maximum dP/dt, mm Hg s^{-1}			
Basal	1648±291	1358±89	0.36
Maximal	1151±174*	945±85*	0.31
Coronary resistance, mm Hg min mL^{-1}			
Basal	28.7±1.9	21.3±1.3	0.03†
Maximal	32.3±3.3*	20.5±2.1	0.01
Angiotensin II			
Heart mass, mg	154±11	173±7	0.15
Heart rate, min^{-1}			
Basal	306±17	325±19	0.51
Maximal	329±20*	358±23	0.55
Maximum dP/dt, mm Hg s^{-1}			
Basal	2281±407	1938±474	0.60
Maximal	1874±303	1867±413	0.99
Coronary resistance, mm Hg min mL^{-1}			
Basal	28.2±3.8	24.8±3.2	0.46†
Maximal	35.2±5.4*	29.4±5.0*	0.44

Values are mean±SEM. P indicates probability of C57BL/6 being different from CD36−/−, obtained with a 2-sample unpaired t test with separate variance (n=8 to 9 hearts per strain).

*P<0.05 compared with the corresponding basal value (paired t test); †P=0.09 when hearts treated with hexarelin and angiotensin II are pooled.

hormone/insulin-like growth factor-I axis seemed to be involved, this effect raised the question about the presence of distinct and specific receptors for GHRPs at the myocardial level.[9] Our approach to identify these putative receptors by covalent binding studies, using a photoactivatable derivative of hexarelin, has led to the discovery of a distinct type of binding sites in cardiac membranes from different mammalian species.[9] Using N-terminal sequencing, the purified photolabeled receptor is identified as CD36, a membrane glycoprotein of M_r 84 000 belonging to the scavenger receptor type-B family of proteins.[19] This receptor is specifically expressed in adipose tissue, platelets, monocytes/macrophages, dendritic cells, and microvascular endothelium.[20,21] The multifunctional character of CD36 has been evidenced by its role in lipid metabolism,[22,23] the recognition and clearance of apoptotic cells,[23] insulin resistance,[24] and the regulation of angiogenesis.[25] Effectively, CD36 expressed in the monocytes/macrophages was reported to contribute to the early phase of the pathogenesis of atherosclerosis through endocytosis of oxidized low-density lipoproteins.[26] This scavenger receptor in combination with thrombospondins and the $\alpha_v\beta_3$ integrin complex was identified as the adhesion molecule on macrophages for the clearance of apoptotic polymorphonuclear leukocytes and for the uptake of apoptotic neutrophils.[23] Its role in mediating the negative modulation of angiogenesis of thrombospondins[25] has also been documented. In the present study, an unexpected vasoactive role of CD36 elicited by hexarelin in the perfused heart model has been demonstrated. The increase of the coronary perfusion pressure induced by hexarelin in the perfused heart model might result from the direct interaction of this ligand with CD36 expressed on membranes of endothelial cells of the microvasculature because the lack of this effect was observed in CD36 knockout mice and in genetically CD36-deficient SHR. This vasoactive response induced by hexarelin is comparable to that of angiotensin II and is correlated with the expression of the scavenger receptor assessed by immunodetection and covalent photoaffinity labeling with the photoactivatable derivative of hexarelin. The signal transduction pathways mediating the vasoconstrictive effect of hexarelin seemed to involve in part L-type calcium channels and protein kinase C.[9] Vasoconstrictor prostanoids were ruled out because the cyclooxygenase inhibitor, indomethacin, was not be able to block the vasoconstriction.[9] Apart from the role of CD36 as a scavenger receptor in foam cell formation and atherogenesis, CD36 is reported for the first time to mediate the coronary vasoconstriction, which may explain the vasospasm seen in hypercholesteremia and atherosclerosis.[27] The cardiovascular effect of hexarelin mediated by CD36 appears to be distinct to that of ghrelin, an endogenous growth hormone–releasing peptide that was reported to feature hypotensive effect with the decrease of the vascular resistance.[28,29] This hemodynamic effect of ghrelin was thought to be mediated by its specific G protein–coupled receptor.[3,4] Taken together, these results emphasize the cardiovascular importance of CD36 for which the development of potential antagonists may be considered.

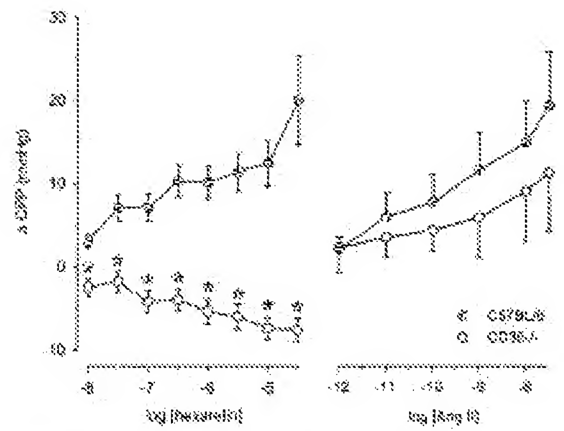

Figure 6. Change in coronary perfusion pressure (CPP) induced by increasing concentrations of hexarelin (left) and angiotensin II (Ang II, right) in hearts from CD36−/− (open circles, n=5) and C57Bl/6 (filled circles, n=7) mice. *Concentrations for which a significant (P<0.05) difference was found between groups (analysis of variance).

Acknowledgments

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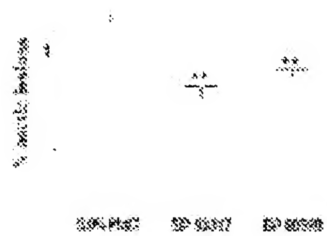
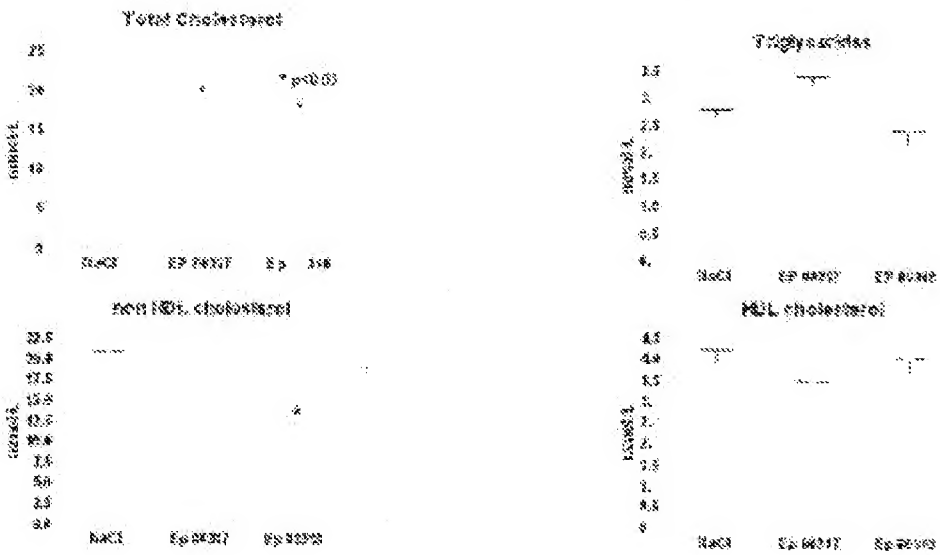
Exhibit 5: Figures illustrating efficacy of EP80318

A chronic treatment with EP 80318 or EP 80317 reduces the percentage of total aortic lesions by 30% and 41%, compared to 0.9% NaCl, respectively



The anti-atherosclerotic effect is paralleled with 31% and 26% reduction of total plasma cholesterol in mice treated with EP 80318 and EP 80317 respectively



Neither triglycerides
nor HDL cholesterol plasma
concentrations is modulated
by either one of the treatments

Curative effect of EP 80318 administered daily to ApoE-null mice fed a high fat high cholesterol diet for 6 weeks (weeks 12-18)